Mechanisms of Inactivation of \( p14^{ARF} \), \( p15^{INK4b} \), and \( p16^{INK4a} \) Genes in Human Esophageal Squamous Cell Carcinoma

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Abstract

The 9p21 gene cluster, harboring growth suppressive genes \( p14^{ARF} \), \( p15^{INK4b} \), and \( p16^{INK4a} \), is one of the major aberration hotspots in human cancers. It was shown that \( p14^{ARF} \) and \( p16^{INK4a} \) play active roles in the p53 and Rb tumor suppressive pathways, respectively, and \( p15^{INK4b} \) is a mediator of the extracellular growth inhibition signals. To elucidate specific targets and aberrations affecting this sub-chromosomal region, we constructed a detailed alteration map of the 9p21 gene cluster by analyzing homozygous deletion, hypermethylation, and mutation of the \( p14^{ARF} \), \( p15^{INK4b} \), and \( p16^{INK4a} \) genes individually in 40 esophageal squamous cell carcinomas (ESCCs) and compared the genetic alterations with mRNA expression in 18 of these samples. We detected aberrant promoter methylation of the \( p16^{INK4a} \) gene in 16 (40%) of the 40 SCC tumor samples, of which 15 (38%) tumor samples contained \( p16^{INK4a} \) methylation, while all but one of the \( p14^{ARF} \) and \( p15^{INK4b} \) methylations were exclusive, whereas all but one of the \( p14^{ARF} \) and \( p15^{INK4b} \) methylations were accompanied by concomitant \( p16^{INK4a} \) methylation. We detected homozygous deletion of \( p16^{INK4a} \) in 7 (17.5%), of \( p14^{ARF} \) in 13 (33%), and of \( p15^{INK4b} \) in 16 (40%) tumor samples. Most deletions occurred exclusively on the \( p15^{INK4b} \) loci. Two samples contained \( p14^{ARF} \) deletion but with intact \( p16^{INK4a} \) and \( p15^{INK4b} \). No mutation was detected in the \( p14^{ARF} \) and \( p16^{INK4a} \) genes.

Introduction

The 9p21 chromosomal band is one of the most frequently altered genomic regions in human cancers (1). Within a short distance of \( \sim 50 \) kb, this region harbors a gene cluster consisting of three genes, \( p14^{ARF} \), \( p15^{INK4b} \), and \( p16^{INK4a} \), all of which have putative tumor suppressor roles (2). In addition to physical proximity, the genomic structures of these genes are remarkably inter-related (Fig. 1). \( p14^{ARF} \) and \( p16^{INK4a} \) transcripts are produced via utilization of a common coding sequence for exons 2 and 3, together with distinct sequences for promoter and exon 1 (3). However, the resulting proteins are completely different because different reading frames are used for the respective translation processes (4). The conservation in mammalian genome of this unique gene structure, usually seen in primitive organisms subject to genome size constrains, suggests either the possible ancient origin or the biological essentialness of the unitary inheritance of these two genes. \( p15^{INK4b} \) is highly homologous to \( p16^{INK4a} \), particularly in exon 2, where they share 91% sequence identity (5), indicating their origination by a gene duplication event. The 9p21 gene cluster is the first of its kind identified in the human genome associated with multiple tumor suppressor activities. Besides its functional importance in regulating cell proliferation, which makes this gene cluster a target of selective inactivation during carcinogenic process, there may be a physical basis underlying its frequent disruption in cancer. It was reported that at least two tightly clustered breakpoints exist within the cluster, the sequence context of which potentially facilitates illegitimate V(D)J recombination activities (6). Furthermore, the promoter regions of all three genes are highly abundant with CpG islands that are susceptible to hypermethylation (7). It is intriguing why presumably important genes are clustered in such a manner that renders high susceptibility to genetic alterations.

Recent studies have revealed that \( p14^{ARF} \) and \( p16^{INK4a} \) play active roles in the p53 and Rb growth-control pathways, respectively (Fig. 1; Ref. 8). \( p16^{INK4a} \) is a cyclin-dependent kinase inhibitor functioning upstream Rb. It can negatively regulate cell cycle progression by preventing the phosphorylation (inactivation) of Rb during G1 phase (9). \( p14^{ARF} \) restrains cell growth by abrogating Mdm2 inhibition of the p53 activity, and therefore facilitates p53 mediated cell cycle arrest and apoptosis (10). It was demonstrated that oncogenic Ras elicits an anti-tumorigenic response mediated by the up-regulation of both \( p14^{ARF} \) and \( p16^{INK4a} \), which in turn activate the tumor suppressors p53 and pRb, respectively (11, 12). Recent study further...
involvement in both pathways, the is observed in most human cancers (1). By virtue of its close stimuli such as TGF-β19) and affected biliary tract cancers, and hereditary melanoma (reviewed in Ref. p15 INK4b is less prominent as a tumor suppressor. In contrast to p16 INK4a, which is activated by intracellular stimuli, p15 INK4b suppresses cell growth in response to extracellular stimuli such as TGF-β (5).

Fig. 1 Genomic organization of the 9p21 gene cluster and schematic description of the involvement of p14ARF, p15INK4b, and p16INK4a gene products in the Rb and p53 antitumorigenic pathways [based on a diagram that appeared in Robertson et al. (7)]. Solid lines, regulatory steps of each pathway; dashed lines, cross-talk between the Rb and p53 pathways.

Inactivation of the Rb and p53 tumor suppressor pathways is observed in most human cancers (1). By virtue of its close involvement in both pathways, the p14ARF–p15INK4b–p16INK4a gene cluster at chromosome 9p21 may be a nexus of the cellular growth-control network, the inactivation of which results in collapse of the tumor suppression system. To date, a vast amount of data has demonstrated multiple types of genetic alterations on the 9p21 region, the prevalence of which varies with the type of tumors. For example, large homozygous deletions are common in head and neck tumors, bladder carcinomas, and malignant gliomas (14–16). Transcriptional-inactivating promoter methylation was common in breast and colon cancer (17, 18). Recently, Robertson et al. (7) identified that the promoter of p14ARF gene is a CpG island and observed its hypermethylation in colon cancer cell lines, which was responsible for the gene inactivation (7). Point mutation and small deletion of the p16INK4a locus was common in pancreatic adenocarcinoma, biliary tract cancers, and hereditary melanoma (reviewed in Ref. 19) and affected p16INK4a exclusively or together with p14ARF (4). Despite the ample evidence of the 9p21 gene cluster as a frequent tumor target, it is not known whether all three genes in the cluster are indiscriminately affected in a tumor and whether all of the genes are uniformly disrupted by the same mechanism.

We reported previously an alteration pattern of the 9p21 region in human ESCC,4 in which p16INK4a was primarily affected by hypermethylation and less frequently subject to deletion, whereas p15INK4b was frequently homozygously deleted and occasionally methylated (20). This pattern is different from reports on other types of cancers identifying p16INK4a as the main deletion target and p15INK4b as a bystander, which possibly play a small part in the tumor suppressor role (2). In light of the recent elucidation of the tumor suppressor role of p14ARF, the first exon of which is only 12 kb downstream of the p15INK4b gene, and the presence of evidence of exclusive deletion of p14ARF-E1β with the retention of both p15INK4b and p16INK4a in T-cell acute lymphoblastic leukemia (21), we suspect that our previous result may indicate p14ARF-E1β as being a primary target for inactivation in ESCCs. In consideration of this point, we thought it is necessary to investigate whether genomic alterations indeed associate with altered gene expressions in ESCCs. In the present study, we constructed a detailed alteration map of the 9p21 gene cluster by analyzing the homozgyous deletion and aberrant methylation of the p14ARF, p15INK4b, and p16INK4a genes individually in 40 resected ESCC samples and analyzed the mRNA expression pattern of the respective genes in 18 frozen samples. We show that suppressed mRNA expression occurred at a high frequency for all three genes in primary ESCCs, and homozygous deletion is the primary cause leading to such inactivation for the p14ARF and p15INK4b genes, whereas aberrant methylation is the main event underlining p16INK4a inactivation.

Materials and Methods

ESCC Specimen Preparation and RNA/DNA Extraction. Eighteen primary ESCC specimens, together with their adjacent normal epithelial tissues, were collected from patients in Linzhou City (formerly Linxian) of northern China, a well-recognized high-risk area for ESCC. The samples were frozen in liquid nitrogen right after surgical resection and were kept in −70°C until processing for RNA/DNA extraction. Adjacent normal epithelia accompanying the tumors were identified through pathological examination and dissected directly from the tissue blocks. All tumor tissues were embedded in OTC and cryosected into 15-μm serial slices under −20°C. H&E staining and histopathological examination were performed on a repre-

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4 The abbreviations used are: ESCC, esophageal squamous cell carcinoma; HD, homozygous deletion; kNN, k-Nearest-Neighbor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
sentative section from each tissue block to identify the tumor region. Tumor tissues were then dissected from the neighboring three consecutive sections. Polyadenylated RNA was extracted from both the normal and tumor tissues using the Rneasy Mini kit (Qiagen, Santa Clarita, CA) according to the manufacturer’s specification. To ensure complete comparability of the analyses at the DNA and RNA levels, all leftover remnants from mRNA preparation were reused for genomic DNA extraction by proteinase K digestion and phenol/chloroform extraction. Another 22 pairs of tumor and normal DNAs from the paraffin-embedded ESCC specimens were directly taken from a sample collection (previously analyzed for \textit{p16 INK4a} and \textit{p15 INK4b} status; Ref. 20) and were further examined for \textit{p14 ARF} molecular alterations.

All of the primers used in this study are listed in Table 1.

**Table 1** Summary of oligonucleotides

<table>
<thead>
<tr>
<th>Primer loci</th>
<th>Analytical purpose</th>
<th>Primer sequence/Genomic site</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{p16\textsuperscript{INK4a}} exon</td>
<td>HD</td>
<td>GAGCAGCATGGAGCCTTC</td>
<td>204</td>
</tr>
<tr>
<td>1\textsubscript{a}</td>
<td></td>
<td>AATTCCTCTGCAAACCTTGGT</td>
<td></td>
</tr>
<tr>
<td>\textit{p16\textsuperscript{INK4a}} exon 2</td>
<td>HD</td>
<td>CACTCTACCCGCACCGGT</td>
<td>222</td>
</tr>
<tr>
<td>\textit{p14\textsuperscript{RRF}} E1\beta</td>
<td>HD and SSCP</td>
<td>ACCCTCGGCGCCATCTAT</td>
<td>177</td>
</tr>
<tr>
<td>1st half</td>
<td></td>
<td>TCCCCAGCTGCACTTTAAGGG</td>
<td></td>
</tr>
<tr>
<td>2nd half</td>
<td></td>
<td>TAGAACACGAAACCTCAG</td>
<td>178</td>
</tr>
<tr>
<td>\textit{p15\textsuperscript{INK4b}} exon 2</td>
<td>HD</td>
<td>ACCGGTGCAATGAGCT</td>
<td>172</td>
</tr>
<tr>
<td>\textit{p16\textsuperscript{INK4a}}</td>
<td>mRNA expression</td>
<td>CAACGCCACGAAATAGTACG</td>
<td>176</td>
</tr>
<tr>
<td>\textit{p14\textsuperscript{RRF}}</td>
<td>mRNA expression</td>
<td>AGCACACACACGGTGT</td>
<td>264</td>
</tr>
<tr>
<td>\textit{p15\textsuperscript{INK4b}}</td>
<td>mRNA expression</td>
<td>GTTTTTGTTTGTTCACTCCC</td>
<td></td>
</tr>
<tr>
<td>\textit{p14\textsuperscript{RRF}} E1\beta</td>
<td>Methylated specific</td>
<td>GTGGAGTTTGGTTTTGGAGGT</td>
<td>160</td>
</tr>
<tr>
<td>\textit{p14\textsuperscript{RRF}} E1\beta</td>
<td>Unmethylated specific</td>
<td>GTGGAGTTTGGTTTTGGAGGT</td>
<td>165</td>
</tr>
<tr>
<td>\textit{p14 ARF} E1\beta</td>
<td>HD and SSCP</td>
<td>AAAACCRCACAGGAAGG</td>
<td></td>
</tr>
<tr>
<td>\textit{p14 ARF} E1\beta</td>
<td>Methylated specific</td>
<td>GAGTTTGGTTTTGGAGGTGG</td>
<td></td>
</tr>
<tr>
<td>\textit{p15 ARF} E1\beta</td>
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<td></td>
</tr>
<tr>
<td>\textit{\beta-Actin}</td>
<td>Internal control</td>
<td>CTGGTCATCCACGAACTA</td>
<td>187</td>
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<tr>
<td>\textit{GAPDH}</td>
<td>Internal standard</td>
<td>TCTCATGGTTTTCCACACCCAC</td>
<td>456</td>
</tr>
</tbody>
</table>

\textsuperscript{a} In methylated- and unmethylated-specific primers, Ts (or As for antisense) that were converted from Cs by bisulfite modification were underlined. Unchanged Cs (or Gs for antisense) were in bold case.

\(5/40\) (12.5%) \(6/40\) (15%) \(16/40\) (40%)

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\(5/40\) (12.5%) \(6/40\) (15%) \(16/40\) (40%)

**Fig. 2** Genomic positions of the PCR targets. The regions that were amplified and their genomic positions for hypermethylation analysis (solid lines) and HD (dashed lines) are shown. The alteration numbers and percentages are shown above or below the lines. The sequence of \textit{p14\textsuperscript{RRF}} was taken from GenBank (accession no. L41934). The sequences of \textit{p16 INK4a} and \textit{p15 INK4b} are as described previously (20).
the primary target. About 100 ng of DNA were used for the
duplex PCR amplification. Forward primers of both target gene
and control were radiolabeled at the 5' end with [γ-32P]dATP.
After 27–29 cycles of PCR, the products were resolved on 6%
denaturing or 8% non-denaturing polyacrylamide gel, depending
on product size difference. After autoradiograph for 4 h, the film
was developed, and the ratio of intensity of the target gene
control was measured and calculated using a computer
imaging system (Image-Pro Plus; Media Cybernetics, Silver
Spring, MD). A HD was scored if the normalized signal intensity
(target/β-actin) in the tumor sample was <25% of that in
the normal epithelium. The threshold was so chosen because of
an estimated upper limit of 20–25% noncancerous cell contamina-
tion in our tumor samples.

Measurement of mRNA Expression of the p14ARF,
p15INK4b, and p16INK4a Genes. cDNA was synthesized using
the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA) with
random priming as recommended in the protocol provided.
Comparative PCRs were performed with the GAPDH gene as
internal standard and one of the gene in the 9p21 gene cluster as
target. PCR cycle numbers (typically 27–30 cycles) were exper-
imentally determined in pilot studies to limit the reaction in the
linear stage. PCR products were resolved on 20% non-denaturing
polyacrylamide gel or 3% agarose gel, and signal intensities
were quantified using a computer image system. The levels of
the gene transcripts were quantified by the ratio of the intensity
of the target signal over the intensity of the GAPDH internal
standard in the same duplex PCR reaction.

Statistical Pattern Recognition and Data Classification.
To establish a reasonable criterion discriminating the different
mRNA expression states, expression level data measured from
all tumor and normal samples for a single gene were subject to
automatic pattern recognition and clustering using the standard
kNN method (22), where the “k” was set at 3. This procedure
was performed independently for each gene of interest. A density-
distribution curve was calculated and plotted using S-PLUS
statistical package on a Sparc-3 Station, and evaluation of nor-
mality of the density distribution was done by comparing the
distribution with a normal distribution using “qqnorm” plot
ments. In the unmethylated-specific PCR product (25N-U, the cloned
fragment as marked under the cloning vector), all of the “C”s were
converted to “T”s (arrow), but in the methylated-specific product (25T-
M), the “C”s in CpG dinucleotides were unchanged because of meth-
ylation, which resisted chemical modification by bisulfite.

Results

Aberrant Methylation of 5′-CpG Islands in 9p21 Gene
Cluster. The methylation status of the p14ARF, p15INK4b, and
p16INK4a CpG islands were investigated in 40 ESCC samples
together with their adjacent normal epithelial tissues. Among
them, 18 were frozen samples and 22 were from a collection of
paraffin-embedded specimens analyzed previously for p15INK4b
and p16INK4a alterations (20); enough DNA remained for anal-
yses of p14ARF alterations. Using methylation-specific PCR
(Fig. 3a), among the 40 samples, we detected aberrant methy-
lization of the p14ARF gene in 6 samples (15%), of the p15INK4b
gene in 5 samples (12.5%), and of the p16INK4a gene in 16
samples (40%). The frequency of the p15INK4b and p16INK4a
methylation is consistent with our previous observation (20).
The aberrant methylation of p14ARF in primary tumors, to our
knowledge, has not been previously reported. Ten of the 16
p16INK4a-methylation cases were observed exclusively on the
p16INK4a promoter region. On the other hand, four of the six
cases with p14ARF methylation and four of the five cases with
p15INK4b methylation occurred in samples in which all three
genes have aberrant methylation. In many cases, in addition to
the presence of methylation-specific PCR signal, a PCR signal
corresponding to the unmethylated DNA sequence could also be
detected from the same samples. The unmethylated signals
might come from stromal cells present between the cancer nests,
which could not be completely removed in our microdissection
procedure. To verify the specificity of the PCR reaction, we
sequenced the PCR products that resulted from both methylated-
specific amplifiers and unmethylated-specific amplifiers and

Fig. 3 Determination of the methylation status of the CpG islands
(using p14ARF analysis as an example). a, methylation-specific PCR
analysis. Two pairs of samples, representing presence of CpG island
hypermethylation in tumor but not in normal DNA (no. 25), or no such
event in either tumor or normal DNA (no. 26), are shown as examples.
CpG methylation is determined by the presence of PCR product amplifi-
cation by methylated-specific primers (M). Lack of methylation is evi-
denced by the presence of only PCR product amplified by unmethyl-
ated-specific primers (U). b, confirmation of methylated-specific PCR by sequencing the PCR frag-
ments. In the unmethylated-specific PCR product (25N-U, the cloned
fragment as marked under the cloning vector), all of the “C”s were
converted to “T”s (arrow), but in the methylated-specific product (25T-
M), the “C”s in CpG dinucleotides were unchanged because of meth-
ylation, which resisted chemical modification by bisulfite.
confirmed that, indeed, only in the unmethylated-specific PCR product was every "C," including those in the CpG dinucleotide, changed to a "T" as a result of chemical modification. In the methylated-specific PCR product, the CpG sequence was not changed to a "T" as a result of chemical modification. In the middle two panels, the PCR products were resolved on 6% sequencing gel. HD of the p14 ARF was performed to generate a 177-bp fragment covering exon 1 of the p14 ARF gene. A 284-bp fragment of the p14 ARF gene transcript, a 428-bp fragment of the GAPDH gene, and p16 INK4a transcript were determined in 18 frozen ESCC samples. The transcriptional regulation of the p14 ARF gene is shown in Fig. 5. The two clusters correspond to minimally overlapped groups of data points and were therefore designated as class I and class II gene expression levels. Class I is tightly clustered with a small mean value and is well approximated by a Gaussian distribution. Class II has a significantly greater mean value and a much broader distribution not strictly Gaussian. Statistically, this suggests the presence of nonrandom regulating factors in samples showing class II mRNA levels but no such mechanisms underlining class I level mRNA expressions. On the average, the mRNA level of class II is ~10 times higher than that of class I. We interpret the two classes as representing distinct states of gene expression; class I corresponds to a basal level, or suppressed state of gene expression, and class II corresponds to active gene expression. We defined the thresholds for the expression levels of class I and class II gene expression. We defined the thresholds for the expression levels of class I and class II gene expression. Class I is defined as basal expression, whereas class II is defined as active expression.

Homologous Deletion of the p14ARF, p15INK4b, and p16INK4a Genes. Gene deletion was examined by differential PCR analysis of the genomic DNA. Duplex PCR amplification was performed to generate a 177-bp fragment covering exon 1β of the p14ARF gene, a 172-bp fragment in the exon 2 of p15INK4b, a 204-bp exon 1α fragment of p16INK4a, or a 222-bp exon 2 fragment of p16INK4a, together with a 187-bp fragment of the β-actin gene as a reference (Fig. 4). The β-actin fragment was amplified in all 40 normal samples, but it was either not detected or had a much lower intensity in 13 tumor samples (32.5%). We presumed that these 13 cases contained a homologous deletion at the first exon of the p14ARF gene, and the low residue signals may come from wild-type stromal and infiltrative cells. Likewise, together with the previously analyzed paraffin samples, homologous deletion of p15INK4b gene was observed in 16 (40%) of the 40 cases. In contrast, a relatively lower percentage of samples showed homologous deletions of p16INK4a. Seven of the 40 samples (17.5%) contained homologous deletion at E2. We performed the homologous deletion analysis for E1α on the 18 frozen samples (nos. 25–62) that had enough DNA for the study. Four of them showed homologous deletion at E1α. The deletion pattern of E1α is the same as that of E2, suggesting that E2 was always codeleted with E1α because of their physical proximity. Only five cases contained homologous deletion in all three loci, and 13 cases had deletion only at the centromeric end of the cluster (E1β-15INK4b loci), whereas exclusive deletion of the p16INK4a at the telomeric end was observed in only two cases. In many cases, E1β of p14ARF was deleted together with p15INK4b. However, two samples (nos. 38 and 920925) contained homologous deletion at p14ARF only.

Mutational Analysis of E1β of the p14ARF Gene. Previously, we analyzed a collection of paraffin-embedded ESCC samples for p16INK4a gene mutation and detected no point mutation in the samples included in the present study (20). We further performed PCR-single strand conformation polymorphism analysis of E1β of the p14ARF gene in the 18 frozen ESCC samples, using methods described previously. We found no E1β mutation in these 18 samples, consistent with the reports from other laboratories (7).

Characterization of p14ARF, p15INK4b, and p16INK4a mRNA Expression. Levels of the p14ARF, p15INK4b, and p16INK4a transcripts were determined in 18 frozen ESCC samples by comparative RT-PCR analysis. A 264-bp fragment of the p14ARF gene transcript, a 428-bp fragment of the p15INK4b transcript, or a 176-bp fragment of the p16INK4a transcript were generated, respectively, with a 456-bp fragment of the GAPDH transcript amplified as the internal standard. Although RT-PCR signals of the genes of interest were detected (at least with a minimal level) in almost all samples, nonparametric classification of the signal intensities (normalized by GAPDH signal) using the kNN method (22) revealed that the mRNA expression level of each of the genes had an apparent two-cluster distribution (Fig. 5). The two clusters correspond to minimally overlapped groups of data points and were therefore designated as class I and class II gene expression levels. Class I is tightly clustered with a small mean value and is well approximated by a Gaussian distribution. Class II has a significantly greater mean value and a much broader distribution not strictly Gaussian. Statistically, this suggests the presence of nonrandom regulating factors in samples showing class II mRNA levels but no such mechanisms underlining class I level mRNA expressions. On the average, the mRNA level of class II is ~10 times higher than that of class I. We interpret the two classes as representing distinct states of gene expression; class I corresponds to a basal level, or suppressed state of gene expression, and class II corresponds to active gene expression. We defined the thresholds for the expression levels of class I and class II gene expression. We defined the thresholds for the expression levels of class I and class II gene expression. We defined the thresholds for the expression levels of class I and class II gene expression. We defined the thresholds for the expression levels of class I and class II gene expression. We defined the thresholds for the expression levels of class I and class II gene expression. We defined the thresholds for the expression levels of class I and class II gene expression. The thresholds for basal levels that correspond to the Gaussian-like class I were simply set to μ + 2σ, whereas because of the spreadness of the distribution of class II, we take μ - 3σ as the lower limit of the active expression state to ensure enough separation between basal and active expression. Between the two thresholds is a range of class II expression.

![Fig. 4](https://example.com/fig4.png) HD of the p14ARF-E1β, p15INK4b, and p16INK4a-E1α, E2 in primary ESCCs determined by duplex PCR. Arrows, band positions of the genes being examined; the remaining band in each gel-panel is the β-actin internal control. In the upper and lower panels, the PCR products were resolved on 6% nondenaturing polyacrylamide gel. HD can be seen in sample 28 for both p15INK4b and p16INK4a. In the middle two panels, the PCR products were resolved on 6% sequencing gel. HD of the p14ARF-E1β is apparent in samples 28 and 38. HD of p16INK4a-E1α can be seen in sample 28 only. P, positive control using human placenta DNA.
Fig. 5 Classification of the mRNA expression levels of the p14ARF, p15INK4b, and p16INK4a genes. Data points representing the measured expression signal intensities of each gene were classified by kNN classifier and were also analyzed using S-PLUS statistical software. The approximate probabilistic-distribution-density curve of the expression levels was plotted for each gene (X axis, expression level), and all of the values of the measured expression levels were marked by * under the curve. It is apparent that the expression levels of each gene fall into two distinct classes. Arrow thresholds for accepting a value as representing active or basal level expressions. Between the two thresholds is the rejection region, values that were considered as indeterminable in the case of expression state. As summarized in Table 2, a great variation of gene expression exists in normal tissues adjacent to the ESCCs. Among all 18 normal samples, 9 and 7 actively expressed the p14ARF and p15INK4b genes, respectively, and for both genes an additional three showed intermediate level (close to the active threshold) expression, whereas basal level expressions of these two genes were less frequent. These seemed to suggest a frequent presence of oncogenic and extracellular growth factor stimuli in the cells close to cancer. However, although presumably also inducible by oncogenic stimuli (11), in most normal cases (14 of 18) the p16INK4a gene displayed only a basal level of expression. Taken together, there was no association in the expression of p14ARF, p15INK4b, and p16INK4a mRNA in the 18 normal samples, suggesting the existence of distinct regulations of each members of the 9p21 gene cluster.

Although the 456-bp GAPDH signal was invariant in all 18 ESCC tumor samples, suppressed mRNA expression was observed in 12 (67%) tumor samples for p14ARF, in 9 (50%) tumor samples for p15INK4b, and in 12 (67%) tumor samples for p16INK4a (Table 1 and Fig. 6). There is one tumor sample (no. 47) containing p14ARF expression at an intermediate level slightly above the threshold of suppression state. Detailed analyses revealed that for all three genes, the suppression of gene expression in tumors had two patterns. For example, among the 12 tumor cases that failed to actively express p14ARF, 9 cases (nos. 25, 36, 38, 42, 43, 46, 47, 48, and 56) contained a high or intermediate level of p14ARF mRNA in the normal epithelia adjacent to the tumors (pattern I), and the remaining 4 cases (nos. 28, 33, 40, and 60) corresponded to a basal level of p14ARF expression in the accompanying normal epithelia (pattern II). Likewise, among the 9 tumor samples that showed suppressed p15INK4b expression, 5 were categorized as pattern I suppression, and 4 were categorized as pattern II suppression. Among the 12 tumor samples that showed suppressed p16INK4a expression, 3 showed pattern I suppression and 9 showed pattern II suppression. Taken together, only 4 of the 18 ESCC samples maintained an elevated level of mRNA expression for all of the three genes. The remaining 14 (77%) cases either showed suppression of all three genes (8 cases) or at least one of the three genes in the tumor (Fig. 7). These results suggested that the 9p21 gene cluster expression is frequently suppressed in ESCC cells.

Correlation Between Gene Expression and Molecular Alterations of the 9p21 Cluster. All promoter hypermethylation and homozygous deletion events detected in the p14ARF, p15INK4b, and p16INK4a genes corresponded to an absence or significant repression of the mRNA expression of the respective genes. Taken together, among the 12 frozen tumor samples showing repression of p16INK4a expression, 8 contained hypermethylation and 4 had deletions of the p16INK4a gene. Whereas among the nine samples with repressed p15INK4b expression, three had hypermethylation and six had homologous deletion. For p14ARF, however, an exact correspondence of altered gene expression to alteration at E1β locus was not observed; among the 12 cases showing repressed expression, 2 had promoter hypermethylation, 6 had E1β deletion, and 4 had no detectable genetic alterations at the E1β locus. Sample 47, which had a close-to-suppression intermediate p14ARF expression, also harbored p16INK4a methylation.
polyacrylamide gels, and products for p14 ARF solved on 3% agarose gels. p14 ARF, p15 INK4b and p16 INK4a possibly exist in these tissues. The p14ARF, p15INK4b and p16INK4a, however, was frequently repressed in ESCC tumor samples; among which, 44% (8 of 18) of the samples showed repression of all three genes. 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 (24). p16INK4a has been widely regarded as the major target of 9p21 deletion. But because of the dual coding capacity of the p16INK4a-p14ARF locus, a deletion occurred on p14ARF exons 2 or 3 could still disrupt p16INK4a. There is evidence in a murine system that much of the tumorigenic phenotype associated with p16INK4a deletion may in fact be attributable to disruption of the p14ARF gene (23). However, whether p14ARF is a primary inactivation target is in debate. Point mutations in E1β are rare. Mutations in E2 of p14ARF and p16INK4a almost exclusively inactivate p16INK4a protein only (25). Deletion of p14ARF is either accompanied by deletion of p15INK4b or p16INK4a or both. Hypermethylation of p14ARF was observed in several colon cancer cell lines, which also contained hypermethylation on p16INK4a (7).

In our study, we found that deletion at the p16INK4a locus (7 of 40) was significantly less frequent than at p14ARF E1β and p15INK4b loci (13 of 40 and 16 of 40, respectively). In fact, most of the p16INK4a deletions (five of seven) only occurred in cases harboring complete 9p21 gene-cluster deletion. These results suggest that p14ARF, rather than the p10INK4a gene, is more likely to be a main target of deletion at 9p21. Although mostly deleted together with p15INK4b, possibly due to their physical proximity, p14ARF-specific deletion was seen in two ESCC samples (nos. 38 and 920925), and such event has also been reported previously (21, 26). Underlining the structural basis of this type of genomic changes, a recent study revealed the existence of tightly clustered breakpoints close to the E1a and E1β loci, and possibly also upstream of p15INK4b (21). There is evidence that the rearrangements in this region may involve illegitimate V(D)J recombinase activity, which could contribute to frequent gene-specific deletions (6). Recent studies suggested the importance of p14ARF as a potent tumor suppressor. Mice lacking p19ARF (the mouse homologue of p14ARF) develop a cancer phenotype (27), p14ARF-null embryonic fibroblasts, that exhibit a high rate of spontaneous immortalization and could be transformed by oncogenic Ras (23). The nature of p14ARF as a bona fide tumor suppressor gene in ESCC also owns to its remarkable frequency of transcriptional inactivation, 12 of 18 ESCCs, among the highest in the 9p21 gene cluster in our study. p14ARF can activate p53 both by neutralizing Mdm2, which destabilizes p53 (10), and by interacting directly with p53 (28) in response to oncogenic stimuli. Therefore, the loss of p14ARF function in tumor cells as observed in our study potentially compromises the p53-mediated cell cycle arrest and apoptotic process therein.

A second deletion hotspot is the p15INK4b locus. Although in most cases p15INK4b deletion was accompanied by a concomitant E1β deletion, 4 of the 16 deletion cases exclusively targeted p15INK4b; p15INK4b has often been considered as an innocent bystander of the deletion at 9p21 (24). Previous evidence of p15INK4b alteration in ESCC has been scarce, and its tumor suppressor role in esophageal carcinogenesis is uncertain. Our observation of frequent and sometimes elusive p15INK4b dele-
tion suggests that p15INK4b may itself be a tumor suppressor gene disrupted during ESCC development. Despite its close linkage and functional similarity to p16INK4a, p15INK4b plays a role of nonredundant cell cycle checkpoint. It is a mediator in the cell cycle control pathway originating from extracellular stimuli such as transforming growth factor β and IFN-α (5, 29). In principle, this pathway is independent of the intracellular pathway mediated by p16INK4a and is equally crucial in maintaining a balanced cell cycle regulation. Tumorigenic transformation in somatic tissues are frequently preceded or accompanied by other cellular abnormalities, such as inflammation or increased proliferation. The growth factors secreted by the inflammatory cells and the increased cellular contact may produce such growth inhibition signal mediated by p15INK4b to preclude cell cycle progression and therefore provide a way of counteracting the transformation tendency. It is possible that inactivation of p15INK4b can desensitize the cell to such extracellular signals and as a result contribute to cancer development. Indeed, evidence of specific inactivation of the p15INK4b gene has been increasing in recent studies in acute lymphoblastic leukemia (30) and other solid tumors (summarized in Ref. 2).

Our observation of relatively infrequent homozygous deletion at the p16INK4a locus is consistent with most previous studies on primary ESCC tumors (31, 32) but disagrees with the results from ESCC cell lines (summarized in Ref. 33). Hayashi et al. (34) suggested that simultaneous loss of p16INK4a and p14ARF expression, which was observed in 11 of 18 frozen tumor samples in our study, is an indicator of homozygous deletion of p16INK4a. However, we found that 8 of the 11 cases actually contained hypermethylation on the p16INK4a promoter. Most of the methylation took place exclusively on the p16INK4a promoter. On the basis of the significantly higher frequency (40%) of hypermethylation on p16INK4a compared with p14ARF (15%) and p15INK4b (12.5%), and its relatively low deletion rate,
we propose that $p16^{INK4a}$ is a primary target of aberrant hypermethylation in ESCC. Our hypothesis of $p16^{INK4a}$ as a primary methylation target can easily explain observations in the previous report of cases that fail to express $p16^{INK4a}$ but express $p14^{ARF}$ readily (34), because $p14^{ARF}$ is controlled by a different promoter (3), which may not be comethylated with $p16^{INK4a}$. The preferential methylation of the $p16^{INK4a}$ promoter may relate to its special local genomic structure, featured by a cluster of breakpoints located just 5' to E1α, the sequence content of which bears the hallmark of V(D)J recombinase activity (6). It was shown that aberrant DNA structures, such as integration intermediates, seem to unleash de novo activity of the mammalian DNA methyltransferase (35). In our study, the sharp difference of deletion rates between the $p16^{INK4a}$ and E1β-$p15^{INK4b}$ loci suggests possible rearrangement hotspot upstream of the $p16^{INK4a}$ locus. Therefore, frequent $p16^{INK4a}$ promoter methylation may be relevant to the frequent rearrangement events upstream of $p16^{INK4a}$. Transcriptional silencing promoter-methylation has been rarely observed for $p14^{ARF}$ and $p15^{INK4b}$ in epithelium-derived tumors. Our observation of both events revealed an alternative mode of $p14^{ARF}$ and $p15^{INK4b}$ inactivation in ESCCs, although at a low frequency. Except for one case with methylation exclusively on $p14^{ARF}$, all methylation events beyond the $p16^{INK4a}$ gene have concomitant $p16^{INK4a}$ methylation, suggesting they have an association with $p16^{INK4a}$ methylation and can also be exclusively targeted.

Although all abnormalities of $p16^{INK4a}$ and $p15^{INK4b}$ expression correspond to an underlying homozygous deletion or hypermethylation of the gene, we were unable to establish a good match between the E1β alterations and inactive $p14^{ARF}$ expression. Specifically, we observed four tumors samples that showed inactive $p14^{ARF}$ expression but contained neither detectable E1β deletion or promoter methylation. Among them, one case (no. 48) can be easily explained by a deletion at $p16^{INK4a}$, because that event also removed part of the $p14^{ARF}$ gene sequence shared with $p16^{INK4a}$. The remaining three cases (nos. 33, 36, and 40), together with a sample (no. 47) with lower intermediate level of $p14^{ARF}$ expression, all appeared to have intact $p14^{ARF}$ gene, except that they all contained hypermethylation in the downstream $p16^{INK4a}$ locus. Gonzalgo et al. (36) have shown in cell lines that hypermethylation of $p16^{INK4a}$ had no effect on the transcription of $p14^{ARF}$. It would be very interesting to determine whether other factors, such as p53, which is stabilized by $p14^{ARF}$ and causes the down-regulation of $p14^{ARF}$ (7), also contribute to $p14^{ARF}$ inactivation.

In summary, our results show that the newly identified growth suppressor $p14^{ARF}$, together with $p15^{INK4b}$, is a primary target of homozygous deletion, whereas $p16^{INK4a}$ is the hypermethylation hotspot in human ESCC. Such a polarity may reflect the presence of sequence-specific elements that favor such alteration or the effect of growth selection during cancer formation. Because recent studies have shown that oncogenic stimuli elicit the antitumorigenic response by up-regulating both $p14^{ARF}$ and $p15^{INK4b}$, which in turn activate the tumor suppressors p53 and pRb, respectively (10, 11), the tumor suppressor role of the 9p21 gene cluster becomes apparent. The unique genomic structure and compact organization of these genes as a cluster may be essential for the highly coordinated regulation in maintaining a balanced Rb and p53 pathway function. Our observation of frequent coinactivation of $p14^{ARF}$ and $p16^{INK4a}$, and even inactivation of the entire 9p21 gene cluster, provides additional evidence for the dysfunction of both Rb and p53 tumor suppression pathways in ESCC development.

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References


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