CDKN2A/p16 Inactivation Mechanisms and Their Relationship to Smoke Exposure and Molecular Features in Non–Small-Cell Lung Cancer

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Introduction: CDKN2A (p16) inactivation is common in lung cancer and occurs via homozygous deletions, methylation of promoter region, or point mutations. Although p16 promoter methylation has been linked to KRAS mutation and smoking, the associations between p16 inactivation mechanisms and other common genetic mutations and smoking status are still controversial or unknown.

Methods: We determined all three p16 inactivation mechanisms with the use of multiple methodologies for genomic status, methylation, RNA, and protein expression, and correlated them with EGFR, KRAS, STK11 mutations and smoking status in 40 cell lines and 45 tumor samples of primary non–small-cell lung carcinoma. We also performed meta-analyses to investigate the impact of smoke exposure on p16 inactivation.

Results: p16 inactivation was the major mechanism of RB pathway perturbation in non–small-cell lung carcinoma, with homozygous deletion being the most frequent method, followed by methylation and the rarer point mutations. Inactivating mechanisms were tightly correlated with loss of mRNA and protein expression. p16 inactivation occurred at comparable frequencies regardless of mutational status of EGFR, KRAS, and STK11, however, the major inactivation mechanism of p16 varied, p16 methylation was linked to KRAS mutation but was mutually exclusive with EGFR mutation. Cell lines and tumor samples demonstrated similar results. Our meta-analyses confirmed a modest positive association between p16 promoter methylation and smoking.

Conclusion: Our results confirm that all the inactivation mechanisms are truly associated with loss of gene product and identify specific associations between p16 inactivation mechanisms and other genetic changes and smoking status.

Key Words: p16, CDKN2A, Inactivation, Homozygous deletion, Methylation, Lung cancer, Adenocarcinoma, Meta-analysis.

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years of diagnosis. We believe lung cancer prognosis can be improved by a combination of early detection with advances in biology and the subsequent implementation of effective targeted therapies such as those directed against EGFR mutations and ALK translocations.

Studies have shown that patients with lung adenocarcinoma often have genetic mutations in EGFR, KRAS, STK11 (also known as LKB1), TP53 (also known as p53), and CDKN2A (also known as p16 or INK4a). Several new potential targets for therapeutic approaches have also been identified recently. A study of these mutations may eventually lead to novel therapeutic applications.

One of the most common genetic alterations in many forms of cancer including lung adenocarcinoma is inactivation of p16. p16 is located at chromosome region 9p21 and is encoded by the CDKN2A gene. In addition to p16, CDKN2A encodes a completely unrelated tumor suppressor protein, ARF, which interacts with TP53. The simple tandem arrangement is complicated by the presence of an additional exon 1β, which is transcribed from its own promoter. The resulting RNA incorporates exons 2 and 3 but specifies a distinct protein because the exons are translated by an alternative reading frame. Thus, although exons 2 and 3 are shared by the two mRNAs, they encode different protein products, p16 and ARF.

p16 is a tumor suppressor, functions as an inhibitor of CDK4 and CDK6, the D-type cyclin-dependent kinases that initiate the phosphorylation of the retinoblastoma tumor suppressor protein RB, and induces cell-cycle arrest. Both alleles must be inactivated before its function is eliminated. Three mechanisms have been implicated in its inactivation: homozygous deletion (HD), hypermethylation in the promoter CpG island (methylation), and point mutation. It has been reported that p16 is frequently inactivated by HD or promoter hypermethylation, and rarely by point mutation in primary NSCLC. Studies demonstrated that the frequency of p16 methylation is significantly higher in lung adenocarcinoma with KRAS mutation, however, the associations between p16 inactivation mechanisms and other common genetic mutations in lung adenocarcinoma such as EGFR and STK11 remain controversial or have never been explored. Smoking has been reported to be associated with p16 methylation and p16 HD was found to be associated with never smokers in some studies, but these findings are inconsistent.

Despite the large number of reports, many of these associations remain controversial, in part because the majority of the reports (1) do not examine all the mechanisms of inactivation, and (2) do not demonstrate that the inactivating mechanism(s) studied were truly associated with inactivation of the gene. Therefore, the aims of the present study were to: (1) examine all the three described inactivation mechanisms of p16 in lung cancer cell lines and tumor samples; (2) demonstrate that our methods of detecting inactivation are truly associated with inactivation; (3) correlate the data with data on tobacco exposure; and (4) correlate inactivation mechanisms with molecular features. Because one of our major interests was correlation with tobacco exposure, we aimed to study sufficient numbers of cancers arising in never smokers. Thus, with a few exceptions, we limited our study to adenocarcinoma as they are by far the most common form of lung cancer arising in never smokers.

**MATERIALS AND METHODS**

**Cell Culture**

Forty NSCLC cell lines were used in the study. Thirty-six of them were lung adenocarcinomas, one was large-cell carcinoma, one was adenosquamous carcinoma, and two were unspecified NSCLC cell lines. Data for the cell lines have been reported in multiple previous studies. In this study, light smokers were defined as patients with less than 15 pack-year history. Heavy smokers were defined as those who had a smoking history of 15 pack-years or more. Refer to Supplementary Table 1 (Supplemental Digital Content 1, http://links.lww.com/JTO/A465) for further information.

**Tumor Samples**

Forty-five tumor samples were included in this study and all of them were obtained from patients with primary lung adenocarcinoma. Institutional review board approval and informed consents were obtained from the patients for molecular analysis of the samples. Among the 45 tumor samples, 29 of them were obtained from smokers, either current or former smokers, and 16 of them were from never smokers. Further details about smoking histories were not available. Refer to Supplementary Table 2 (Supplemental Digital Content 1, http://links.lww.com/JTO/A465) for further information.

**DNA, RNA Extraction, and cDNA Synthesis**

Genomic DNA was obtained from cell lines and tumor samples by standard phenol–chloroform extraction or by using the DNeasy Tissue Kit (QIAGEN, Alameda, CA). Total RNA was extracted from cell lines using the RNeasy Plus Mini Kit (QIAGEN). The cDNA was prepared by reverse transcription of RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol.

**p16 Inactivation Methodology**

Because of the multiple methods used to determine and confirm inactivation, and their applications to tumors and cell lines, these methods are summarized in Supplementary Table 3 (Supplemental Digital Content 1, http://links.lww.com/JTO/A465) and detailed below.

**Homozgyous Deletions**

HDs of p16 in cell lines were detected using SYBR green (Bio-Rad Laboratories, Inc., Irvine, CA) real-time quantitative polymerase chain reaction (qPCR) and single-nucleotide polymorphism (SNP) which will be discussed later in the article. The sequences of the primers used were forward: 5′-GTGAAGCCATTGCAGAACT-3′ and reverse: 5′-TTCTTTCAATCGGGGATGTC-3′. Both primers recognize sequences located in intron 2. The reactions were performed in a Bio-Rad DNA Engine Thermal Cycler (Bio-Rad Laboratories, Inc.). The thermal cycling conditions were set to 2 minutes at 50°C, 10 minutes at 95°C, followed by
of both genomic DNA and cDNA were performed dependent on material availability, and all the reactions were performed using the Applied Biosystem GeneAmp PCR System 9700 instrument. For DNA sequencing, exon 1 was amplified by PCR using the following primers: forward: 5'-GAAGAAAGAGGAGGGCT-3' and reverse: 5'-GCGCTACCTGATATCCAATTTC-3'. PCR was performed for 42 cycles consisting of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 2 minutes. Exon 2 was amplified using the following primers: forward: 5'-AGCTTCTTTTCGTCATGC-3' and reverse: 5'-GGAAGCTCTCAGGTACCAATTTC-3'. PCR was performed for 42 cycles consisting of denaturation at 94°C for 1 minute, annealing at 48°C for 1 minute, and extension at 72°C for 2 minutes. For amplification of exon 3, the following primers were used: forward: 5'-CTTGCATTTGAGCAACC-3' and reverse: 5'-GGTTCTGGAATTTGCTACAC-3'. PCR was performed for 44 cycles consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. For cDNA sequencing, the following primers were used: forward: 5'-CGAAGGGAAGAAGGAG-3' and reverse: 5'-TTCTCAGACCTCTCTGTT-3'. PCR was performed for 44 cycles consisting of denaturation at 94°C for 1 minute, annealing at 50°C for 90 seconds, and extension at 72°C for 2 minutes.

Exome sequencing was performed on an Illumina GAIIx platform using standard procedures. Postsequence data was processed with Illumina Pipeline software v 1.7.

**Expression Analysis**

Taqman gene expression assay for p16 was used to quantitatively detect p16 mRNA levels according to the manufacturer's protocol (Applied Biosystems).

RNA-seq was also used to measure p16 transcript expression levels. One microgram of total RNA per sample was poly-A tail purified, fragmented, and adapter ligated using Illumina TruSeq RNA sample prep kit version 2, following the instructions of manufacturers. Samples were multiplexed four to a lane on the Illumina HiSeq 2000 using the Single Read version 3 flowcell and underwent a 100 base pair single-end run using TruSeq version 3 SBS kits. Sequencing was performed at the Southern California Genotyping Consortium, University of California, Los Angeles, California. Sequence data was read using Illumina OBC (off-line base caller) software, was trimmed to remove adapter reads, and aligned to the National Center for Biotechnology Information (NCBI). GrCh37 (hg19) transcriptome using CLCbio Genomics Workbench version 5.1. Reads with more than three mismatches to the reference genome (poor alignment) or more than 10 identical copies (PCR bias) were discarded. Coverage was determined by read density over the gene body and used to estimate expression levels.

Protein expression of p16 was assessed by mass spectrometric (MS) analysis. The collection of whole cell extracts was performed as described in a previous study. Protein digestion and identification by liquid chromatography-MS/MS were performed as described previously. Three International Protein Indexes (IPIs) were identified for CDKN2A in which
IPI0001560 and IPI00651662 correspond to p16 whereas IPI00478390 corresponds to ARF.

**Mutation Status of EGFR, KRAS, STK11, TP53, and RB**

The 40 cell lines and 45 tumor samples in our study were examined for mutations in exons 18 to 21 of the EGFR gene and exons 1 and 2 of the KRAS gene by genomic PCR and direct sequencing as described in our previous studies.\(^{27-29}\) Mutations in exons 1 to 9 of the STK11 gene were detected by cDNA sequencing in both cell lines and tumors. The following primers were used: forward: 5’-GAAGGGAAGTCGGAACACAA-3’ and reverse: 5’-CCCTGGCTATGCAGGTAC-3’; forward: 5’-ATGGAGTAATCGTGGTGTGGG-3’ and reverse: 5’-CAG CGGGAATGTTCTTTCT-3’; forward: 5’-TTTGAGATCGGACAT CGGG AAAG-3’ and reverse: 5’-AACCGCGAGCAAGAC TGAG-3’. Mutations in exons 1 to 11 of the p53 gene were detected by cDNA sequencing only in cell lines. The following primers were used: forward: 5’-GCTTTCCACGACGTTGGC-3’ and reverse: 5’-GCTTTCCACGACGTTGGC-3’; forward: 5’-GTTCTGCTTGGATGGCCA-3’; forward: 5’-GTTCTGCTTGGATGGCCA-3’; forward: 5’-GTCTGGGCTTCTCTTGGGCCA-3’; forward: 5’-GTCTGGGCTTCTCTTGGGCCA-3’; forward: 5’-GTTCTGCTTGGATGGCCA-3’; forward: 5’-GTTCTGCTTGGATGGCCA-3’. The detailed method was described in the section on HD.

**Copy Number Analysis of CCND1, CDK4, and CDK6**

The copy numbers of CCND1, CDK4, and CDK6 in 40 cell lines were detected using SYBR green real-time PCR (qPCR). The sequences of the primers used for CCND1 were forward: 5’-GCGGAGGAGAACACACATGC-3’ and reverse: 5’-GGCTGTCTGACACATGC-3’. The detailed method was described in the section on HD.

**Statistical Analysis**

Frequencies of all three p16 inactivation mechanisms in each mutational group (EGFR, KRAS, and STK11 mutation), and smoking group were compared using Fisher’s exact test. Some of these genetic mutations are known to be associated with each other, smoking status, sex, and ethnicity. To prevent the potential confounding effect of multiple variables, a multivariate logistic regression model was used to analyze the association of specific genetic mutations or smoking status with the various mechanisms of p16 inactivation. Results with \(p\) value less than 0.05 were regarded as statistically significant.

**Meta-Analysis**

Literature published from 2001 to May 2012 in PubMed database were screened by using key words “p16 or CDKN2A or INK4A” and “smoking or tobacco,” and “promoter methylation” or “HD” or “mutation” or “inactivation” to determine the impact of smoking. Key words “never smoker” or “never smoke,” or “never smoking” were used instead of “smoking” or “tobacco” in the meta-analysis for never smokers. Only articles providing raw data and published in English were selected. The meta-analyses were performed using R (www.r-project.org) with the Rmeta package. A Woolf’s test was performed to identify heterogeneity among publications. The random-effects model was used for heterogeneous studies whereas the fixed-effects model was applied if the studies were homogeneous.\(^{30}\) To prevent one of the studies from dominating the results by contributing a larger number of samples or a big effect size, we adopted the leave-one-out strategy by removing data from each study one at a time and detected how the overall odds ratio (OR) had changed. The overall OR was found to remain significantly greater than one no matter which study was removed in both meta-analyses, hence none of the selected studies dominated the result.

**RESULTS**

**Multiple Methodologies for the Detection of p16 Inactivation**

p16 can be inactivated by HD, hypermethylation of promoter CpG islands, or point mutations. In this study, we investigated the inactivation mechanisms of p16 in 40 NSCLC cell lines and 45 NSCLC tumor samples. As shown in Table 1, the frequency of p16 inactivation was higher in cell lines (75%) compared with that of tumor samples (38%), and this has been observed in previous studies.\(^{14}\) Despite the higher frequency of inactivation in cell lines, no evidence of difference in the proportions of p16 inactivation mechanisms was found between cell lines and tumor samples. Hence, the results for tumors and cell lines are presented individually and were also combined to increase the sample size for our analyses (Table 1).

HD was the most frequent mechanism of inactivation in both cell lines (53%) and tumors (59%), as detected by copy number changes (SNP arrays). Representative examples of

**TABLE 1.** Frequency and Mechanisms of p16 Inactivation

<table>
<thead>
<tr>
<th></th>
<th>Frequency of p16 Inactivation (%)</th>
<th>Mechanisms of p16 Inactivation</th>
<th>HD (%)</th>
<th>Methylation (%)</th>
<th>Mutation%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lines</td>
<td>30/40 (75)</td>
<td></td>
<td>16/30 (53)</td>
<td>10/30 (33)</td>
<td>4/30 (13)</td>
</tr>
<tr>
<td>Tumors</td>
<td>17/45 (38)</td>
<td></td>
<td>10/17 (59)</td>
<td>4/17 (24)</td>
<td>3/17 (18)</td>
</tr>
<tr>
<td>Combined</td>
<td>47/85 (55)</td>
<td></td>
<td>26/47 (55)</td>
<td>14/47 (30)</td>
<td>7/47 (15)</td>
</tr>
</tbody>
</table>

HD, homozygous deletion.
HDs are shown in Figure 1. For cell lines, qPCR was used to confirm the results of the SNP arrays.

The methylation status of p16 was assessed by quantitative MSP analysis. p16 methylation was present in 30% (33% for cell lines and 24% for tumors) of samples with p16 inactivation. We also confirmed these results of 38 cell lines (data were not available for cell lines PC-9 and DFCI032) by Illumina Infinium Human Methylation 450K BeadChip. Probe cg13601799 for detecting exon one of p16 (E1α) and probe cg03079681 located in exon one of p14ARF (E1β) were selected (Fig. 2A). Only cell lines with methylated p16 detected by MSP analysis showed high levels of methylation using probe cg13601799, and these cell lines are not methylated in p14ARF exon 1 (probe cg03079681; Fig. 2B). Among them, NCI-H969 showed partial methylation of p16 as indicated by its β value. Of interest, there are some values for β scores in the cell lines carrying an HD of the CDKN2A locus. These probably result from deletions in the region, which are artifactually affecting the β value resulting from a comparison of the unmethylated and methylated probes. Thus the results do not reflect the correct methylation status of the cell lines. We also examined the methylation status of NSCLC cancers in the TCGA database (Fig. 2C). We found that p16 methylation was more frequent in squamous cell carcinomas (36%) than in adenocarcinomas (17%), which is consistent with published reports.

The p16 mutational status was determined by direct sequencing for cell lines and tumors. p16 mutations were present in 15% (13% for cell lines and 18% for tumors) of

![Image](image_url)

**FIGURE 1.** HDs of p16 in lung adenocarcinoma tumors and cell lines. Affymetrix SNP six arrays were used to generate copy number profiles for lung adenocarcinoma cell lines and clinical lung tumors. Allele-specific copy numbers were derived using the allele-specific copy number workflow in PGS software. Copy number profiles for lung cancer cell lines were generated using a panel of 72 nondiseased HapMap individuals as a baseline whereas matched nonmalignant lung tissue was used as a reference for defining copy number changes in tumors. Arrows indicate region of the p16 gene. A, Focal HD of p16 in the lung adenocarcinoma cell line, NCI-H1944. B, Lung adenocarcinoma tumor illustrating neutral p16 copy number state. C, Focal HD of p16 in a lung adenocarcinoma tumor. Both HDs in (A) and (C) occur in a background of single copy loss on chromosome 9p. Copy number states in regions of deletion do not drop right to zero because of tumor cell heterogeneity, aneuploidy, and/or the presence of nonmalignant cells that contribute to array signals. Maximum (red) and minimum (blue) alleles are defined by copy number state. Each dot represents the smoothed copy number for 30 adjacent array probes. Copy number states (HD, single copy loss, or copy neutral) are indicated. Images were generated in PGS. Arrows indicate location of p16 gene at chromosome 9p21.3. HD, homozygous deletion; PGS, Partek Genomics Suite.
p16 inactivation. Additionally, the results were confirmed by next-generation sequencing in the cell lines.

In summary, the results obtained by different methods designed to measure the same type of inactivation (HD, methylation, or sequence mutation) were concordant with each other in every case.

Association between p16 Inactivation and Its mRNA and Protein Expression

As shown in Figure 3A (with details presented in Supplementary Table 4, Supplemental Digital Content 1, http://links.lww.com/JTO/A465), the concordance between all the three inactivation mechanisms (HD, methylation, point mutations) and RNA and protein expression were excellent. With the exception of one partially methylated cell line (NCI-H969), all HD and methylated cell lines showed absent RNA or protein expression. With the exception of PC-9, cell lines with point mutations expressed low or absent levels of mRNA and protein. Thus 28 of 30 cell lines (93%) demonstrating any method of inactivation lacked p16 expression. By contrast, nine of 10 (90%) of wild-type (WT) lines expressed varying levels of mRNA expression and eight of nine (89%) had protein expression.

For representative cell lines we confirmed our results using RNA-Seq data. We selected four NSCLC cell lines (NCI-H2228, NCI-H1975, NCI-H23, and NCI-H522) to represent the different statuses of p16 (HD, mutation, methylated, and WT, respectively), as illustrated in Figure 3B. Unlike ARF (E1β), which was detected in the other three cell lines except for the p16 HD cell line, p16 expression (E1α) was only detected in the p16 WT cell line. These data indicate that all three p16 inactivation mechanisms (HD, methylation, point mutation) affect p16 expression levels.
Expression analysis of the CDKN2A locus in NSCLC cell lines. A, Heatmap shows RNA and protein expression levels in cell lines carrying no detectable alteration of the CDKN2A locus (WT), DNA methylation of the CpG island in exon 1a (p16INK4a) (Meth), a point mutation in p16INK4a (Mut), or an HD of the locus. RNA expression was determined by qPCR. The relative mRNA levels of p16 were compared with that of nonmalignant immortalized HBECs. Protein expression was determined by mass spectrometry. *Indicates Rb mutants. B, RNA-Seq data for the CDKN2A locus for representative cell lines. The different splice variants arising from the locus are indicated. Genome-wide coverage was similar for all four lines. Peaks detected are shown to scale. No signal was detected in the HD line (NCI-H2228), p14ARF mRNA, but not p16INK4a was detected in the Mut (NCI-H1975) or Meth (NCI-H23) lines; mRNA containing exon 1a (p16INK4a) was only detected in WT line (NCI-H522). HD, homozygous deletion; NSCLC, non–small-cell lung carcinoma; HD, homozygous deletion; HBECs, bronchial epithelial cells; WT, wild-type; qPCR, quantitative polymerase chain reaction.

**P16 Inactivation in Lung Adenocarcinoma with Other Genetic Mutation**

Because EGFR, KRAS, and STK11 are frequently mutated in lung adenocarcinomas,9,11 we investigated the association between mutational status of these genes and p16 inactivation. Among the 40 cell lines, 10 were EGFR mutants, 15 were KRAS mutants, and 15 were STK11 mutants. As previously reported, EGFR and KRAS mutations were mutually exclusive.25 Mutations of EGFR and STK11 were also found to be mutually exclusive in the 40 cell lines. KRAS and STK11 mutations coexisted in seven cell lines. Of the 45 tumor samples, 14 had EGFR mutations, 21 had KRAS mutations, and 20 had STK11 mutations. Again, the EGFR and KRAS mutations were mutually exclusive. Six of the tumor samples had coexisting STK11 and EGFR mutations, whereas seven tumor samples had mutations in both STK11 and KRAS. The detailed information of mutational status of each sample is provided in Supplementary Tables 1 and 2 (Supplemental Digital Content 1, http://links.lww.com/JTO/A465).

Overall p16 inactivation was detected with comparable frequencies regardless of EGFR, KRAS, and STK11 mutational status. Figure 4 and Supplementary Table 5 (Supplemental Digital Content 1, http://links.lww.com/JTO/A465) show a summarization of the data. Among all EGFR mutants, p16 was mostly inactivated by HD (42% combined from cell lines and tumor samples, 50% in cell lines, and 36% in tumor samples), and the remainder demonstrated p16 inactivation by point mutation (13% combined, 20% in cell lines, and 7% in tumor samples). None of the EGFR-mutated cases demonstrated p16 promoter methylation, which has been identified as a common mechanism of p16 inactivation in lung adenocarcinomas in this as well as in a number of other studies.9,12,25 Our analysis revealed that EGFR mutations and p16 methylation were mutually exclusive in all cases, although our sample size was modest. By contrast KRAS mutations were positively correlated with p16 methylation (for cell lines, p = 0.025; for tumors, p = 0.025; for combined, p = 0.003). We did not note any significant relationships between STK11 or p53 (data not shown) mutations and mechanisms of p16 inactivation.

Other variables including smoke exposure, sex, and ethnicity have been reported to be associated with specific mutations. For instance, EGFR mutation is more prevalent in women, Asians, and never smokers whereas KRAS and STK11 mutations were more often found in smokers. Given these known relationships, it is plausible that p16 inactivation...
mechanisms may also be associated with specific clinical features in addition to the molecular features described above. To decipher the effects of multiple clinical and genetic variables, a multivariate logistic regression model was used to determine the associations between particular genetic mutation and p16 inactivation mechanisms. The results were consistent with our initial univariate analyses described above.

We also investigated genetic alterations of other components of the pathway. Although 75% NSCLC cell lines contained p16 inactivation, only two of 40 (5%) had RB mutations (Fig. 5 and Supplementary Table 6, Supplemental Digital Content 1, http://links.lww.com/JTO/A465). We also analyzed the copy number changes of cyclin D1, CDK4, and CDK6 in these cell lines. Low-level gains were present in 20% to 25% of the lines (Fig. 5, Supplementary Table 6, Supplemental Digital Content 1, http://links.lww.com/JTO/A465), whereas high-level amplifications were rare.

**P16 Inactivation in Smokers and Never Smokers**

We failed to find a relationship between smoking status and frequency or method of inactivation of the p16 gene (Fig. 4, Supplementary Table 5, Supplemental Digital Content 1, http://links.lww.com/JTO/A465). Because there are contradictory reports on the relationship of smoking status and p16 methylation, we performed meta-analyses to investigate the relationship of smoking status and p16 methylation as well as with any mechanism of inactivation.11,12,19–21,30–49

**Meta-Analysis of Smoke Exposure and p16 Methylation**

Promoter methylation of p16 is reportedly associated with smoking in some studies but not in others. To address these variable findings, we performed a meta-analysis in addition to analyzing our own data. A total of 23 studies containing 1903 smokers and 982 nonsmokers, were selected as described in the Materials and Methods section. A Woolf’s test was performed to test for heterogeneity among the studies selected, and the results showed that the studies were heterogeneous ($\chi^2$ test = 39.29; df = 23; $p$ = 0.018). A random-effects model was therefore applied and the overall OR from the meta-analysis was 1.99, which was significant ($p < 0.05$) with 95% confidence interval between 1.48 and 2.72. Therefore, our meta-analysis based on the published literature confirmed a positive association between p16 promoter methylation and smoking (Fig. 6).

**Meta-Analysis Smoke Exposure and Other p16 Inactivation Mechanisms**

Although the association between promoter methylation and smoking has been extensively studied, little has been done to explore the potential association between smoke exposure and other p16 inactivation mechanisms. We performed a second meta-analysis to evaluate the association between smoke exposure and other p16 inactivation mechanisms.
exposure and other methods of \( p16 \) inactivation. Five studies that contained data on HDs and/or point mutations with smoking history, were selected. There were total 305 smokers and 99 nonsmokers included in these studies. The Woolf’s test showed that the studies were homogeneous (df = 4; \( p = 0.2680 \)), and hence a fixed-effects model rather than random-effects model was applied. The meta-analysis did not identify any significant association between never smoking and any of the \( p16 \) inactivation mechanisms (Fig. 7), although it did reveal a positive trend of \( p16 \) HD among never smokers. The overall OR was 0.61 (\( p = 0.1068 \)) with 95% confidence interval between 0.338 and 1.11. These results corroborated the findings of our study.

DISCUSSION

To achieve the goals of our study with confidence, we used multiple methodologies to study \( p16 \) inactivation and to confirm inactivation by RNA and protein expression. Reassuringly, the data from each of these methodologies gave concordant results, confirming the accuracy of our data and conclusions of this comprehensive study.

We found \( p16 \) inactivation in 75% of cell lines, mainly via HD (53%) or methylation (33%), and occasionally by point mutations (13%). Because the \( p16 \) gene forms a crucial component of the RB growth regulatory pathway, we investigated genetic alterations of other components of the pathway. Only 5% of cell lines had RB mutations. We also analyzed the copy number changes in these cell lines. Low-level gains of cyclin D1, CDK4, and CDK6 were present in 20% to 25% of the cell lines whereas high-level amplifications were rare. Thus, \( p16 \) inactivation was the major perturbation of the RB pathway noted in NSCLC cell lines.

Recent studies have shown the association between \( p16 \) promoter methylation and \( KRAS \) mutation in lung adenocarcinoma. However, the association between \( p16 \) inactivation mechanisms and other common genetic alterations in lung adenocarcinoma is much less understood or has never been explored. In this study, we demonstrated that \( p16 \) inactivation occurred at similar frequencies regardless of the mutational status of \( EGFR, KRAS, \) and \( STK11 \) in lung adenocarcinoma; however, the patterns of \( p16 \) inactivation were significantly different, depending on the mutational status of these genes.
We also confirmed the link between *KRAS* mutation and *p16* promoter methylation, and showed that *EGFR* mutation and *p16* methylation were mutually exclusive. The negative and positive associations of *p16* promoter methylation with *EGFR* and *KRAS* mutation, respectively, and the similar frequencies of *p16* inactivation in both groups indicated the differences in the evolvement of epigenetic *p16* alterations in these groups; despite the differences, it is clear that inactivation of the *p16* gene is involved in the pathogenesis of both *EGFR*- and *KRAS*-mediated lung cancer.

More recently, *STK11* has been identified as being frequently mutated in lung adenocarcinoma and could play an important role in lung cancer differentiation and metastasis. However, the relationship between *STK11* mutation and *p16* inactivation has never been investigated. Our results showed no significant correlations between *STK11* and *p16* inactivation. Because *STK11* is usually inactivated by HDs, its true incidence may be underestimated. As with HD of *p16*, we examined *STK11* by multiple means (sequencing, RNA expression, copy number) and are confident that these genes are involved in different pathways and play different roles in development of lung adenocarcinoma. Furthermore, our meta-analyses confirm the modest correlation between *p16* methylation and smoking, and the trend of higher frequencies of *p16* HD among never smokers. These findings support the concept that tumors arising in never smokers are driven by distinct molecular mechanisms in lung adenocarcinoma.

In conclusion, our results demonstrate that *p16* inactivation occurs at similar frequencies regardless of the mutation status of *EGFR*, *KRAS*, and *STK11* in lung adenocarcinoma. However, the patterns of inactivation mechanism differ significantly depending upon the genetic mutation present. We also confirm that *p16* methylation is linked to *KRAS* mutation and is mutually exclusive with *EGFR* mutation. Our results indicate that these genes are involved in different pathways and play different roles in development of lung adenocarcinoma.

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