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Advances in Brief

Codeletion of *p15* and *p16* Genes in Primary Non-Small Cell Lung Carcinoma[1]

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Abstract

Chromosome band 9p21 is deleted frequently in non-small cell lung carcinoma (NSCLC), and the *p15* and *p16* cyclin-dependent kinase-4 inhibitor genes map within this deletion region. Recent studies demonstrated deletion of *p15* and *p16* in NSCLC metastases and cell lines, suggesting a role for these genes in NSCLC progression. We now report *p15* and *p16* copy number, as determined by fluorescence *in situ* hybridization with a P1 contig, in 18 primary NSCLCs. Codeletion of *p15* and *p16* was found in 15 of 18 NSCLCs, and 1 of the 3 tumors with normal *p15* and *p16* copy number had a nonsense mutation in exon 2 of *p16*. We conclude that *p15* and *p16* are deleted and/or mutated in most primary NSCLCs. Two observations, however, support the involvement of at least one additional tumor suppressor gene on chromosome 9. These observations are: (*a*) the large size (>100 kb) of most NSCLC *p15/p16* deletions; and (*b*) the absence of exon 2 mutations in most retained NSCLC *p15* and *p16* alleles.

Introduction

Cytogenetic deletions involving chromosome bands 9p13–p22 have been reported in at least 50% of NSCLCs[3] (1–4), and a critical 9p deletion region has been defined in NSCLC through loss of allelic heterozygosity studies using cancer cell lines. Homozygous deletions of the *IFNA/IFNW* and *D9S171* loci were found in several NSCLCs, suggesting a consensus deletion site centromeric to the IFN gene cluster at chromosome bands 9p21–p22 (5–7). These data indicate that a tumor suppressor gene at 9p21–p22 contributes to neoplastic transformation in many NSCLCs. The cyclin-dependent kinase-4 inhibitor genes, *p15* and *p16*, both map to 9p21, and deletions and/or mutations of these putative tumor suppressor genes have been reported recently in NSCLC metastases and cell lines (8–10). Although homozygous deletions of *p15* and *p16* have been detected in cell lines from a wide variety of cancers, including NSCLC (9–14), such deletions have not been observed in primary NSCLCs. The lack of homozygous deletions in primary NSCLCs supports a proposed role of *p15* and/or *p16* loss in tumor progression. However, it is important to note that homozygous deletions were assayed in primary tumor specimens using PCR approaches, and these approaches might be confounded by the presence of nonneoplastic stromal, endothelial, and/or epithelial cells. In the present study, we determined *p15* and *p16* copy number in primary NSCLCs by FISH. Cases that retained *p15* and *p16* alleles were sequenced to determine the presence of inactivating point mutations in either gene. The combined approach of FISH and DNA sequencing demonstrated deletion and/or mutation of *p15* and *p16* in 16 of 18 NSCLCs.

Materials and Methods

Tumor Specimens. Eighteen primary NSCLCs and companion nonneoplastic lung tissues were obtained from biopsy and lobectomy specimens at Brigham and Women's Hospital. One-half of each specimen was processed immediately for cytogenetic analysis, whereas the remaining one-half was frozen at −80°C.

Cytogenetic Analyses. Cell suspensions were obtained from the fresh tumor specimens using a combination of mechanical mincing and enzymatic disaggregation (15), and cell suspensions were then cultured as described previously (15). Metaphase cells were harvested after 24–48 h in culture; harvesting conditions, slide-making, and chromosome banding were as described previously (15). Approximately 20% of the harvested metaphase cells were analyzed cytogenetically from each case. The remaining metaphases were stored in 3:1 methanol:acetic acid (−80°C) for subsequent FISH studies.

FISH Analyses. Metaphase chromosome spreads were dropped on clean glass slides and were then treated with RNase A (100 μg/ml) in 2X SSC (pH 7.0) at 37°C for 1 h, followed by pepsin digestion (50 μg/ml in 0.01 M HCl) for 10 min at 37°C and postfixation with 10% buffered formalin for 2 min. The slides were then washed twice in 70% ethanol for 2 min and stored in 70% ethanol at 4°C overnight prior to denaturation in 70% formamide-2X SSC (1× SSC 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) at 70°C for 2–3 min. Hybridization was with P1 clones 1063 and 1069 (11), which comprise a 100-kb contig including both *p15* and *p16*. DNA was isolated from these clones using conventional alkaline lysis large-scale methods (16) after culture in Terrific Broth with isopropyl-1-thio-β-D-galactopyranoside. One μg each of 1063 and 1069 DNA were labeled by nick translation with digoxigenin-11-dUTP, purified over G-50 fine Sephadex columns, and ethanol coprecipitated with 4 μg Cot-1 DNA and 4 μg herring sperm DNA. This *p15/p16* contig probe was then dissolved in 32 μl of 50% formamide, 10% dextran sulfate, and 2X SSC, and 8 μl of the resulting mixture was denatured for 5 min at 75°C, preannealed for 30 min at 37°C, and quenched on ice. One μl of a biotinylated pericentromeric chromosome 9 probe *D9Z1* (Oncor, Gaithersburg, MD) was mixed with 2 μl of 50% formamide, 10% dextran sulfate, and 2X SSC, denatured for 5 min at 75°C, and quenched on ice. The *p16* and *D9Z1* probes were then mixed together and hybridized to tumor preparations at 37°C overnight. Slides were washed once in 0.5X SSC (pH 7.0) at 72°C for 5 min and twice in PN buffer (PN is 0.1% NP40 in a mixture of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 to give pH 8.0) at room temperature for 1 min. Detection was at 37°C using: (*a*) FITC-avidin and rhodamine anti-digoxigenin (Oncor) for 40 min; (*b*) rabbit anti-sheep antibody (Oncor) for 30 min; (*c*) rhodamine anti-rabbit antibody (Oncor) for 30 min; (*d*) biotinylated anti-avidin D (5 μg/ml, pH 8.0; Vector, Burlingame, CA) for 10 min; and (*e*) FITC-avidin DCS (5 μg/ml, pH 8.0; Vector) for 10 min. Oncor reagents were used according to the manufacturer's protocol, and each incubation was followed by three washes in room temperature PN buffer for 2 min each. Slides were counterstained with 0.1 μg/ml 4′,6-diamidino-2-phenylindole in antifade solution and were evaluated using a triple band-pass Texas Red/FITC/4′,6-diamidino-2-phenylindole filter (Chroma, Brattleboro, VT). Two hundred metaphase cells were evaluated from each specimen, and the copy number of *p15/p16* and *D9Z1* was determined for each cell.

DNA Sequence Analyses. Genomic DNA was isolated from frozen tissue or fixed cell suspensions according to standard methods (16). Primers for PCR and sequencing were based on published sequences (11). Primers for *p15* exon 2 were: 89F, 5′-TGAGTTTAACCTGAAGGTGG-3′; 50R, 5′-GGGTGGGAAATTGGGTAAG-3′; and sequencing, P15F1, 5′-CCACCCTGGCTCTGACC-3′. Primers for *p16* exon 2 were: 42F, 5′-GGAAATTGGAAACTGGAAGC-3′; 551R, 5′-TCTGAGCTTTGGAAGCTCT-3′; and sequencing,

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[3] The abbreviations used are: NSCLC, non-small cell lung carcinoma; FISH, fluorescence *in situ* hybridization.

16F, 5′-CCCTGGCTCTGACCATTCTG-3′. PCR reactions were performed in 25 μl containing 10–50 ng genomic DNA, 0.2 mM of each deoxynucleotide, 0.5 μM primers, 1% DMSO (for *p15*) or 5% formamide (for *p16*), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, and 1 unit Taq DNA polymerase (GIBCO BRL). Exon 2 was amplified (after denaturing at 95°C for 5 min) by 35 cycles at 94°C for 45 s, 56°C for 60 s (*p15*) or 40 s (*p16*), and 72°C for 1 min. The last cycle was followed by an additional incubation at 72°C for 7 min. One to 2 μl of PCR reaction was used for direct sequencing. Sequencing reactions were performed with the fmol DNA Sequencing System (Promega) as described by the manufacturer, except that 5% DMSO was included in the reaction and the cycle number was increased to 45.

Results

Cytogenetic Aberrations. Clonal cytogenetic aberrations were identified in the metaphase cell harvests from each primary tumor. Seventeen tumors had complex chromosome aberrations (five or more clonal aberrations), whereas the one tumor with bronchoalveolar histology (ST91–304) had a noncomplex karyotype: 47,XY,der(1)t(1;14)(p11;q11),−14,+mar1,dmin. All tumors except ST90–387 (squamous cell carcinoma) and ST91–304 (bronchoalveolar carcinoma) had deletions and/or translocations involving chromosome band 9p21 (data not shown).

FISH Analyses. Hybridization efficiencies for the *p15/p16* contig and the *D9Z1* probe were evaluated through FISH against normal mesothelial cells and normal lymphocytes. The *p15/p16* contig and *D9Z1* were cohybridized in these studies, and dual-probe detection was as described for the tumor specimens. Two hundred mesothelial cells (400 expected alleles) were evaluated in slides from three separate FISH experiments, and only 9 *p15/p16* regions were not visualized (hybridization efficiency, 391 of 400; 98%). The hybridization efficiency for *D9Z1* in these normal mesothelial cells was 396 of 400 (99%). Hybridization efficiencies for the *p15/p16* contig and *D9Z1* in lymphocytes were 388 of 400 (97%) and 394 of 400 (99%), respectively.

Percentages of primary NSCLC cells with *p15* and *p16* deletion are listed in Table 1. Partial deletion indicates cells with only one copy of the *p15/p16* region (Fig. 1A), as well as cells with relative deletions of *p15/p16* (fewer copies of *p15/p16* than *D9Z1*). Relative deletions were noted in aneuploid tumors that had three or more copies of the chromosome 9 centromere (*D9Z1*) but fewer copies of the *p15/p16* contig. Complete deletion indicates total absence of *p15* and *p16* loci (Fig. 1*B*). To avoid overinterpretation of incomplete hybridization, NSCLCs were not scored as having *p15/p16* deletion unless 10% of cells had complete or partial loss of *p15* and *p16*. Weak *p15/p16* contig FISH signals, suggestive of incomplete deletion within the contig, were not observed in any tumor. Thus, there was no evidence for selective deletion of either *p15* or *p16*.

Table 1 *p15 and p16 aberrations in 18 primary NSCLCs*

Case	Age/Sex	Histological type[a]	% *p15/p16* deletion		Exon 2 mutation	
			Partial	Total	*p15*	*p16*
ST89-152	72/M	Large cell	24	0	W	W
ST90-096	50/M	Adeno	2	1	W	W
ST90-106	39/M	Large cell	39	5	ND	ND
ST90-126	62/M	Adeno	77	5	W	W
ST90-190	53/M	SCC	0	91	ND	ND
ST90-196	48/M	SCC	4	52	ND	ND
ST90-206	43/F	Large cell	16	47	W	W
ST90-211	59/M	Adeno	81	0	W	W
ST90-223	65/M	Adeno	34	0	W	W
ST90-294	57/M	Adeno	3	82	ND	ND
ST90-338	75/F	Large cell	18	0	W	W
ST90-341	61/M	SCC	76	4	W	M[b]
ST90-387	60/M	SCC	3	0	W	M[c]
ST90-444	35/F	Adeno	62	0	ND	ND
ST91-304	47/M	Bronchoalv	0	69	ND	ND
ST91-312	43/F	Adeno	17	43	W	W
ST92-157	72/M	Adeno	9	84	ND	ND
ST93-500	61/M	Adeno	3	0	ND	ND

[a] Adeno, adenocarcinoma; SCC, squamous cell carcinoma; Bronchoalv, bronchoalveolar; ND, not done; W, wild type.
[b] Codon 75, CAC(His) → AAC(Asn).
[c] Codon 72, CGA(Arg) → TGA(stop).

Of the 18 primary NSCLCs, 15 cases (83%) had total and/or partial *p15* and *p16* deletion (Table 1). Eight NSCLCs had partial loss of the *p15/p16* contig but had few or no cells with total *p15/p16* loss, whereas two cases (ST90–206 and ST91–312) had a mixed deletion pattern, including substantial populations with total and partial deletions. Five NSCLCs had total loss of *p15/p16* but did not have a second population with partial deletion. The *p15/p16* deletions were associated with cytogenetic deletions of chromosome band 9p21 in 14 of 15 tumors; a bronchoalveolar carcinoma (ST91–304) had normal chromosome 9 homologues cytogenetically but had total deletion of *p15* and *p16*.

Evaluation of Intragenic Mutations. Exon 2 of both *p15* and *p16* was sequenced in 10 NSCLCs that retained at least one *p15/p16* region. Eight cases were not sequenced because of total *p15/p16* deletion (five cases) or lack of frozen tumor and nonneoplastic tissues (three cases). Analysis of nonneoplastic companion lung tissues revealed no sequence polymorphisms or germline mutations in the 10 cases that were sequenced. One squamous cell carcinoma (ST90–341) with partial deletion of *p15* and *p16* had a missense mutation of *p16* at codon 75 (CAC→ AAC), and a squamous cell carcinoma with no deletion of *p15* and *p16* (ST90–387) had a nonsense mutation of *p16* at codon 72 (CGA→ TGA; Table 1; Fig. 2).

Discussion

Deletions and inactivating point mutations of the *p15* and *p16* cyclin-dependent kinase-4 inhibitor genes are found in a wide variety of cancers (11–14, 17–19). The proteins encoded by *p15* and *p16* inhibit progression through the G_1 phase of the cell cycle, and deletion or functional inactivation of *p15* and *p16* is expected to promote cell proliferation. Initial studies of *p16* revealed homozygous deletions in many tumor cell lines (11, 12), but subsequent studies demonstrated few *p16* mutations or homozygous deletions in primary tumors (17, 20–22). Among one series of 75 varied primary tumors with loss of allelic heterozygosity at 9p, only 2 had somatic *p16* mutations (20). These data suggest that *p16* might not be the primary target of most 9p21 deletions in uncultured solid tumors. However, homozygous deletion appears to be an important mechanism of *p15* and *p16* inactivation in some tumors (20, 23), and such deletions are challenging to detect in primary tumors containing substantial populations of nonneoplastic cells.

Two recent studies demonstrated that mutations and/or deletions of *p15* and *p16* in a subset of NSCLCs, whereas such aberrations were uncommon in small cell lung carcinomas (9, 10). Washimi *et al.* (9) found homozygous deletions of both *p15* and *p16* in 6 of 20 NSCLC cell lines. This group also demonstrated that homozygous deletion of *p15* and *p16* was not merely an *in vitro* event; homozygous deletion of *p15* and *p16* was confirmed in microdissected, uncultured neoplastic cells from a metastatic tumor that had been used to establish one of the *p15/p16*-deleted cell lines. However, this study did not address the question of *p15* and/or *p16* aberrations in primary NSCLCs. Okamoto *et al.* (10) detected homozygous *p16* deletion by Southern blot analysis in 4 of 22 metastatic NSCLCs but in none of 25 primary NSCLCs. In addition, concordant homozygous deletions of both *p15* and *p16* were found in 7 of 14 NSCLC cell lines. These findings suggested that deletions of *p15* and *p16* might be a late event in tumor progression. However, it is also possible that enrichment of tumor

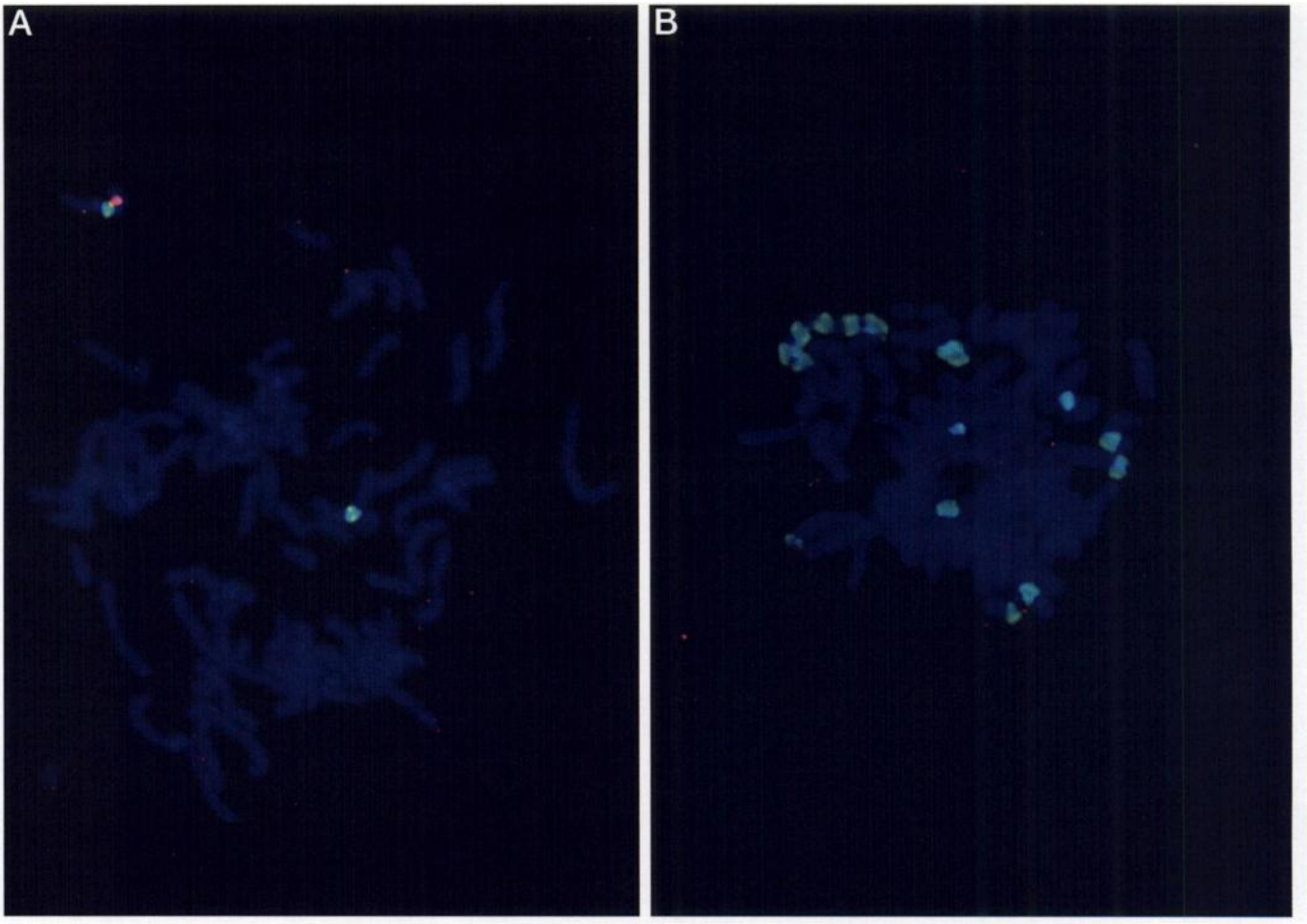

Fig. 1. Dual-color FISH evaluation of *p15* and *p16* (rhodamine detection, *red*) and *D9Z1* chromosome 9 pericentromeric classical satellite (FITC detection, *green*). Partial deletion of *p15* and *p16* is seen in a metaphase cell from adenocarcinoma ST90–444 (*A*); 62% of cells from this carcinoma had partial deletion of *p15* and *p16*. Total deletion of *p15* and *p16*, accompanied by marked amplification of *D9Z1*, is seen in a metaphase cell from squamous cell carcinoma ST90–190 (*B*); 91% of cells from this carcinoma had total deletion of *p15* and *p16*.

cells in metastases and in cell line specimens enables detection of homozygous deletions that were obscured by the substantial nonneoplastic cell populations in primary NSCLCs.

Because most primary NSCLCs contain large numbers of nonneoplastic stromal cells, we studied *p15* and *p16* deletion using FISH methodology that enabled extremely sensitive evaluation of the *p15/p16* region on a cell by cell basis. The sensitivity of this approach is underscored by the high hybridization efficiency of the *p15/p16* contig in nonneoplastic lymphocytes and mesothelial cells. Other indications of the hybridization efficiency using this contig are the FISH results in three NSCLCs that lacked *p15/p16* deletion; *p15/p16* probe hybridization efficiencies in ST90–096, ST90–387, and ST93–500 were 98, 98.5, and 98.5%, respectively. Fifteen of 18 primary NSCLCs (83%) were lacking *p15* and *p16* alleles from at least one chromosome 9 homologue, and 7 cases had substantial cell populations with total loss of *p15* and *p16*. The potential importance of *p15* and/or *p16* deletion was highlighted in our study by the finding of total *p15* and *p16* loss in one bronchoalveolar carcinoma that had cytogenetically unremarkable chromosome 9 homologues (ST91–304). The deletions in this cases were too small to resolve cytogenetically, and it is likely that *p15*, *p16*, and/or a closely neighboring gene were the target of the deletions.

Exon 2 of *p15* and *p16* accounts, respectively, for 63 and 68% of coding sequences in these genes, and the large majority of somatic mutations in *p15* and *p16* have been localized to this exon. We

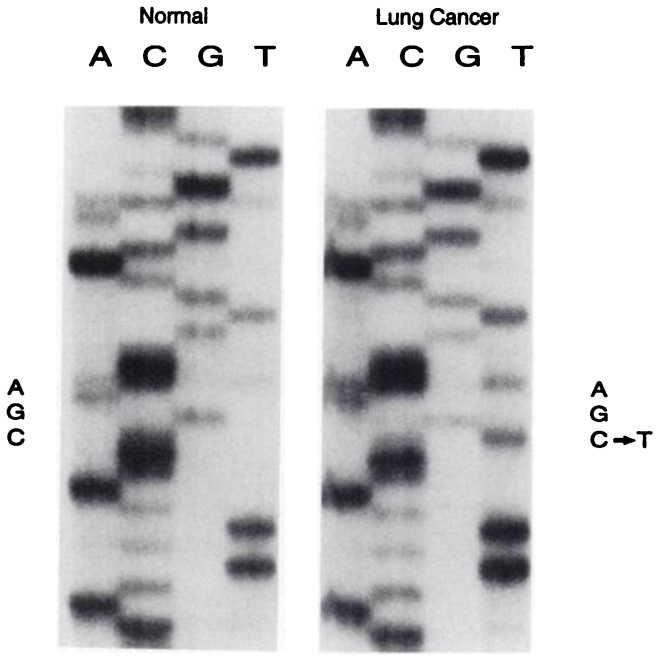

Fig. 2. Sequencing analysis showing nonsense mutation in *p16* (codon 72) from squamous cell carcinoma (ST90–387). This case was one of three NSCLCs that lacked physical deletion of *p15* and *p16*.

sequenced exon 2 of *p15* and *p16* in 10 NSCLCs that retained at least one copy of the *p15/p16* region. A nonsense mutation at *p16* codon 72 was found in one of three cases that lacked *p15* and *p16* deletion, and a missense mutation at *p16* codon 75 was found in one of seven cases with partial deletion of *p15* and *p16*. Nonsense mutations of *p16* codon 72 have been described in several types of cancer (13, 14), and mutations involving *p16* codon 75 have also been reported previously (14, 22). In summary, our findings indicate definite deletions and/or mutations of *p15* and *p16* in 16 of 18 primary NSCLCs. These findings suggest that aberrations of *p15*, *p16*, and/or neighboring gene(s) are important events in primary NSCLCs that likely occur prior to metastatic spread.

The FISH approach described herein demonstrated intratumoral genetic heterogeneity for *p15* and *p16* copy number in two NSCLCs (ST90–206 and ST91–312). Both tumors had one population with partial deletion of *p15* and *p16* and another population with total loss of these genes. We have observed similar genetic heterogeneity in primary malignant mesothelioma (24), and such heterogeneity is in keeping with the proposed role of *p15* and/or *p16* in NSCLC progression *in vivo* and *in vitro* (10, 13). The biological implications and the mechanisms responsible for this heterogeneity are unclear, however. It is possible that loss of a single *p15* or *p16* allele confers proliferative advantage in NSCLCs, whereas subsequent loss of the remaining allele enables tumor progression. Another explanation, perhaps more likely, is that some 9p deletions in NSCLC target *p15*, *p16*, and at least one additional tumor suppressor gene. In this progression-associated model, the first event might be inactivation of one allele of the third tumor suppressor locus by point mutation; the second event might be deletion of all three alleles (*p15*, *p16*, and third locus) from the remaining wild-type chromosome 9 homologue; the cell would now have partial deletion of *p15* and *p16* with total inactivation of the third locus; the third event might be deletion of *p15* and *p16* from the chromosome 9 homologue that already bears a point mutation of the third tumor suppressor locus. This hypothetical mechanism gains support from a number of studies that indicate that at least one additional tumor suppressor locus on chromosome 9 maps outside the *p15* and *p16* region (18, 25–27).

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