Notes

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Differential Inactivation of CDKN2 and Rb Protein in Non–Small-Cell and Small-Cell Lung Cancer Cell Lines

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Background: **The CDKN2 gene encodes the human cyclin-dependent kinase 4 inhibitor. This inhibitor protein is believed to be a tumor suppressor that plays an essential role in cell cycle regulation. One half of all cancer cell lines and one fourth of lung cancer cell lines examined to date contain homozygous deletions (i.e., both alleles lost) of CDKN2. However, the relative frequency of homozygous CDKN2 deletions in non–small-cell lung cancers (NSCLC) and in small-cell lung cancers (SCLC) has not been determined. Inactivation or loss of another tumor suppressor encoded by the retinoblastoma gene (the Rb protein) is more common in SCLC than in NSCLC.** ***Purpose:*** **We measured the frequency of homozygous CDKN2 deletions in 77 NSCLC and in 93 SCLC tumor cell lines. In addition, possible associations were explored between CDKN2 gene loss, the presence or absence of Rb protein, and the clinical status of lung cancer patients.** ***Methods:*** **DNA was isolated from each tumor cell line and from the primary tumor and normal tissue of one NSCLC patient. Sequences corresponding to exons 1 and 2 of the CDKN2 gene were amplified by use of the polymerase chain reaction, and the resulting amplification products were analyzed by agarose gel electrophoresis and DNA blotting. Genomic DNA blotting was also used to evaluate CDKN2 gene deletions. The frequency of homozygous CDKN2 loss and the presence or absence of functional Rb protein (reported previously) in the cell lines were compared.** ***Results:*** **Homozygous deletion of CDKN2 was detected in 18 (23%) of 77 cell lines established from patients with NSCLC, compared with one (1%) of 93 cell lines established from patients with SCLC ($P<.001$). No CDKN2 gene loss was observed in the normal tissue of an NSCLC patient whose tumor cell line showed homozygous deletion of the gene; however, the primary tumor from this patient had evidence of CDKN2 loss. Homozygous CDKN2 deletion was detected in 13 (28%) of 46 tumor cell lines from patients with stage III or stage IV NSCLC, compared with zero of 10 tumor cell lines from patients with stage I or stage II NSCLC. Coincident loss of CDKN2 genes and functional Rb protein was rarely observed (in two of 135 cell lines).** ***Conclusion:*** **The frequency of homozygous CDKN2 gene deletion in NSCLC cell lines is greater than that observed for any other known, or candidate, tumor suppressor gene.** ***Implication:*** **Further study of the role of CDKN2 gene alteration in the pathogenesis of NSCLC is needed. [J Natl Cancer Inst 87:756-761, 1995]**

Deletions and translocation of chromosomal region 9p21 have been detected in different cancers and cancer cell lines, including melanomas (*1*) and lung cancers (*2,3*). Analysis of the deleted segments in this chromosomal region from tumor cell lines identified a locus that contains the human cyclin-dependent kinase 4 inhibitor (CDK4I) gene, also referred to as the p16 or multiple tumor suppressor 1 gene (MTS1) (*4,5*). This gene will be referred to hereafter as CDKN2, the name designated by the Nomenclature Committee of the Human Genome Organization. The CDKN2 gene product inhibits the kinase activity of the cyclin-dependent protein kinase 4 (CDK4)–cyclin D complex by a reversible, noncovalent-binding interaction that blocks the ability of CDK4 to phosphorylate the Rb protein in vitro (*6,7*). Phosphorylation of Rb allows cells to progress through the G_1 phase of the cell cycle (*7*). The absence of CDK4 inhibition has been predicted to be associated with the uncontrolled growth of cancer cells. Recent reports (*4,5*) demonstrated that the CDKN2 gene was homozygously deleted in approximately one half of cancer cell lines examined, ranging from a high of 82% of astrocytomas to 0% of colon cancers and neuroblastomas. In addition, approximately one fourth of lung cancer cell lines examined had homozygous deletions of the CDKN2 gene (*4,5*).

Human lung cancers can be divided into two major categories that have distinct histologic, clinical, and genetic features: small-cell lung cancer (SCLC) and non–small-cell lung cancer (NSCLC) (*8,9*). SCLC tends to present as a disseminated cancer that is not amenable to surgical resection and is typically treated with systemic chemotherapy and chest radiotherapy (*8*). In contrast, NSCLC is typically treated with the local measures of surgical resection or chest radiotherapy and is not as effectively controlled with systemic chemotherapy (*9*). In addition, abnormalities of the retinoblastoma gene have been shown to be more common in tumor cell lines from patients with SCLC than in those from patients with NSCLC (*10-12*). In 90% of the samples studied (*10-17*), SCLCs lacked functional Rb protein. In contrast, 15%-30% of NSCLC

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**See* "Notes" section following "References."

primary lesions and tumor cell lines examined were deficient in Rb (*10,18,19*). If the retinoblastoma and CDKN2 gene products are involved in the same pathway that suppresses the formation of cancers, mutations in the cognate genes would not be predicted to occur in the same cancer. Therefore, the CDKN2 gene is predicted to be inactivated more commonly in NSCLC than in SCLC, because the retinoblastoma gene is more commonly inactivated in SCLC than in NSCLC.

We studied 170 SCLC and NSCLC cell lines established from patients with clinical information to determine how many of the lines contained homozygous deletions of the CDKN2 gene (one common mechanism of CDKN2 alteration). Information was also available on Rb protein expression levels in these cell lines (*10*). As a result, we determined the number of cancer cell lines with abnormalities of the Rb protein and homozygous deletions of the CDKN2 gene, and we studied the nature of the relationship between abnormalities of these two cell cycle control molecules in both the SCLC and the NSCLC cell lines. We also explored possible associations between CDKN2 gene loss and the clinical status of lung cancer patients.

Patients and Methods

Patient Information

Patients with SCLC and NSCLC were evaluated, assigned a stage[1], and treated on approved protocol studies at the NCI-Navy Medical Oncology Branch (*20,21*). Written informed consent was obtained from each patient, and each protocol was approved by the local institutional review boards in accordance with an assurance filed with and approved by the U.S. Department of Health and Human Services.

Tumor Cell Lines Plus DNA and RNA Preparation

Tumor cell lines were established from patient biopsy specimens by use of techniques previously described (*22-24*). Biopsy specimens were identified as having been obtained from patients prior to the initiation of treatment or after the administration of chemotherapy or radiotherapy. Tumor cell lines were categorized as SCLC or NSCLC (adenocarcinoma, squamous cell carcinoma, or large-cell carcinoma). The tumor cell lines were grown in RPMI-1640, HITES (*25*), or ACL4 (*26*) medium supplemented with fetal calf serum. Lung cancer cell lines were harvested during log-phase growth, and DNA and RNA were prepared from the cell lines by previously described techniques (*27*).

CDKN2 Deletion

Polymerase chain reactions (PCRs) were carried out with 100 ng of genomic DNA as the template and oligonucleotide primers specific for either exon 1 (2F and 1108R) or exon 2 (45F and 551R)-(*4*) of the CDKN2 gene. Reaction mixtures (10 µL), containing 5% dimethyl sulfoxide, were prepared with reagents included in the Gene Amp kit (Perkin-Elmer Corp., Norwalk, Conn.). A Perkin-Elmer Cetus 9600 thermocycler was used for 40 cycles at 94 °C for 15 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds. PCR products were analyzed by electrophoresis in a 1.8% agarose gel stained with ethidium bromide. The absence of a band of the predicted size (388 base pair [bp] for exon 1 and 500 bp for exon 2) was interpreted as evidence of a homogygous deletion of that exon in the template DNA. Amplification reactions that yielded fragments of the predicted size were repeated at least two additional times. PCR products of the predicted size (388 bp for exon 1 and 500 bp for exon 2) were confirmed to contain CDKN2 gene sequences by means of Southern blot analysis (*27*) with labeled oligonucleotide probes (CTGGATCGGCCTCCGACCGT for exon 1 and CCTCTCTGGTTCTTTCAATCGGG for exon 2). The presence of intact DNA in the PCR mixtures was confirmed by amplification of a 536-bp segment of the beta-hemoglobin (also known as beta-globin) gene, which is located on chromosome 11 (*28*). Deletion of the CDKN2 gene was confirmed by Southern blot analyses of genomic DNA.

The Southern blot analysis of genomic DNA (10 µg) was performed as previously described (*29*) after digesting the DNA with the restriction endonuclease *Eco*RI. Cloned, radiolabeled CDKN2 exon 1 or exon 2 genomic fragments served as the probes. The final wash prior to exposing the blots to x-ray film was performed in 0.1× standard saline citrate with 0.1% sodium dodecyl sulfate at 50 °C for 20 minutes. Relative amounts of DNA loaded per lane were determined by including a keratinocyte growth factor (KGF) first exon probe in the hybridization solution. The KGF probe hybridizes to a 3.5-kb *Eco*RI fragment located on chromosome 15 (*29*). Quantitation of the signals obtained from the Southern blots was performed with a PhosphoImager and Image Quantitation Software version 3.3 (Molecular Dynamics, Sunnyvale, Calif.).

Northern blot analysis of 20 µg of total RNA obtained from lung cancer cell lines was performed as previously described (*27*) with a CDKN2 full-length complementary DNA probe (*4*).

Statistical Methods

Using the two-tailed chi-squared test, Fisher's exact test, or Mehta's Fisher exact test as appropriate (*30*), we determined the association between CDKN2 and other factors such as stage, Rb protein status, and histologic type of lung cancer. If complete information was available on more than one specimen per patient, data from the earliest obtained specimen were used; otherwise, the data from the specimen with the most complete information were used. Survival duration was calculated from the date of diagnosis for patients with NSCLC and from the start of chemotherapy for patients with SCLC until the date of last follow-up or death. The probability of survival as a function of time was calculated by the Kaplan–Meier method (*31*), and the significance of the difference between pairs of Kaplan–Meier curves was determined by the Mantel–Haenszel method (*32*).

Results

The CDKN2 gene was homozygously deleted in 18 (23%) of 77 tumor cell lines from patients with NSCLC compared with one (1%) of 93 cell lines established from patients with SCLC (Table 1; $P<.001$). Eighteen of the 19 cases of homozygous deletion involved both exons 1 and 2 of the CDKN2 gene. For example, no amplification products were generated for either exon from the DNA of NSCLC cell lines NCI-H647, NCI-H460, NCI-H1648, and NCI-H125 and from the SCLC cell line NCI-H1417 (Fig. 1). However, beta-globin gene PCR products were obtained from all lung cancer cell lines and normal tissues studied, indicating that amplifiable DNA was present in each PCR reaction. The squamous cell carcinoma-derived cell line NCI-H226 contains a deletion of exon 1, but not of the more telomeric exon 2 (data not shown). Southern blot analyses of the PCR products and genomic DNAs from all 19 lung cancer cell lines with putative homozygous deletions were performed to confirm the absence of fragments bearing exons 1 and 2, or, in the case of NCI-H226, fragments bearing exon 1 (data not shown).

Normal tissue was available from one (NCI-H647) of the 18 patients with NSCLC whose tumor cell lines had homozygous deletions. Both exons of the CDKN2 gene were present in the normal tissue of this patient (*see* N-1, Fig. 1). As indicated above, no CDKN2 signal was obtained when genomic DNA from NCI-H647 cells was subjected to Southern blot analysis. A 4.0-kb *Eco*RI fragment containing the first exon of the CDKN2 gene was detected in a Southern blot containing DNA isolated from the primary tumor (NSCLC-1) and the normal tissue (N-1) of this individual (Fig. 2) (*4,5*). However, the intensity of the autoradiographic signal obtained for the 4-kb fragment derived from tumor DNA was measured to be 47% of that obtained for the corresponding fragment from normal tissue DNA. When a radiolabeled exon 2 frag-

Table 1. CDKN2 deletions and Rb protein* in tumor cell lines from patients with lung cancer

	CDKN2 homozygous deletions/No. of patients (%)†	
NSCLC‡	18/77 (23)	
Adenocarcinoma	11/41 (27)	
Large-cell carcinoma	2/11 (18)	
Squamous cell carcinoma	2/6 (33)	
SCLC	1/93 (1)	
	CDKN2 present	CDKN2 absent
NSCLC cell lines, n = 66§		
Rb present	41	16
Rb abnormal	8	1
SCLC cell lines, n = 69‖		
Rb present	8	0
Rb abnormal	60	1

*The status of Rb protein in these cell lines has been previously reported (*10*), except for NCI-H1417 that has no Rb protein (Kaye FJ: personal communication).

†$P<.001$. Comparison of the frequency of CDKN2 deletion in NSCLC versus SCLC was performed with the chi-squared test.

‡Nineteen of the cell lines designated as NSCLC have not been subclassified, so the number of NSCLC cell lines studied was greater than the total of the three histological subtypes of NSCLC.

§$P = .43$. Comparison between Rb and CDKN2 was performed by use of Fisher's exact test.

‖$P = 1.00$. Comparison between Rb and CDKN2 was performed by use of Fisher's exact test.

ment was used to probe the same tumor and normal tissue DNAs, a 53% relative signal intensity was obtained.

In addition, we studied messenger RNA (mRNA) from 28 of the lung cancer cell lines (six NSCLC and 22 SCLC cell lines) by northern blot analyses (data not shown). The three NSCLC cell lines with homozygous CDKN2 gene deletions (NCI-H460, NCI-H1648, and NCI-H1869) did not contain detectable CDKN2 mRNA; similarly, no CDKN2 mRNA was detected in samples from the SCLC cell line NCI-H1417. Two NSCLC cell lines (NCI-H157 and NCI-H1385) and one SCLC cell line (NCI-H1238) did not exhibit evidence of CDKN2 gene deletion and did not produce detectable amounts of CDKN2 mRNA. Twenty-one lung cancer cell lines (20 SCLC and one NSCLC) retained both exons of the CDKN2 gene in their DNA, and they expressed a 1.4-kb mRNA.

The most commonly identified lesion of a specific gene or gene product in cell lines established from patients with SCLC is the loss of functional Rb protein, observed in 88% of the tumor cell lines analyzed (Table 1). One NSCLC cell line (NCI-H2228) and one SCLC cell line (NCI-H1417) were found to lack Rb protein and to also contain homozygous deletions of the CDKN2 gene. None of the eight SCLC cell lines that exhibited normal Rb protein in western blot analysis (*10*) had homozygous deletion of the CDKN2 gene. Thus, deletion of the CDKN2 gene does not appear to be a frequent lesion in tumor cell lines from patients with SCLC.

In contrast, deletions of the CDKN2 gene occurred frequently in tumor cell lines from patients with NSCLC. Homozygous deletions were present in 28% of the tumor cell lines that had apparently normal Rb protein, evaluated on the basis of western blot analysis (Table 1). Forty-one (62%) of 66 of the NSCLC cell lines studied had normal Rb protein and both exons of the CDKN2 gene were present.

Homozygous CDKN2 deletions were found at roughly the same frequency in the three histological subtypes comprising the NSCLCs (Table 1; $P = .80$). None of the lung cancer cell lines established from the 10 patients with stage I or II NSCLC had homozygous deletions of the gene, in contrast to 13 (28%) of the 46 tumor cell lines established from patients with stage III or IV NSCLC ($P = .095$). The survival of patients with stage III or IV NSCLC was not statistically different for the 13 patients whose tumor cell lines had homozygous deletions of the CDKN2 gene compared with the 33 patients whose tumor cell lines did not (Fig. 3).

The therapy given to patients with NSCLC did not appear to influence the incidence of homozygous CDKN2 deletions. Five (28%) of 18 patients with NSCLC who had received either radiotherapy or chemotherapy before obtaining tumor tissue for the establishment of a cell line had homozygous deletions of CDKN2 compared with nine (22%) of 41 patients who had not received either radiotherapy or chemotherapy ($P = .74$)

Discussion

The identification of the CDKN2 gene on chromosome 9p21, which is deleted in a wide variety of cancers, has allowed a molecular analysis of this locus in 170 SCLC and NSCLC cell lines. Homozygous deletion of the CDKN2 gene is more common in lung cancer cell lines from patients with NSCLC than in cell lines from patients with SCLC. Our findings are consistent with previous results from cytogenetic analyses and loss of heterozygosity studies that indicated that NSCLC cells had more abnormalities at this locus than SCLC cells (*2,33,34*). The patients in our study with early stage NSCLC (stage I or II) did not have homozygous deletions of the CDKN2 gene.

Deletions of the CDKN2 gene do not appear to be only an artifact of cell culture. Allelic loss at chromosome 9p21 has been described previously in cancers obtained directly from patients with NSCLC (*3*). In our study, the presence of only about one half the amount of CDKN2 hybridization signal from the Southern blot of DNA extracted from the actual tumor (NSCLC-1) that gave rise to the tumor cell line NCI-H647 is consistent with the following: 1) a tumor consisting of either approximately equal numbers of homozygously deleted tumor cells and normal cells, 2) a nearly pure population of hemizygously deleted tumor cells, or 3) a mixture of all three cell types. Because stromal cells are required for in vivo tumor growth, at least a fraction of the cells in NSCLC-1 were homozygously deleted for the CDKN2 gene. Homozygous deletion of CDKN2 was not present in the normal tissue of the patient from whom the NSCLC-1 tumor was isolated and the NCI-H647 cell line was derived (Fig. 1).

Recent reports have provided conflicting information about the existence of point mutations in the CDKN2 gene in primary NSCLCs. DNA sequence

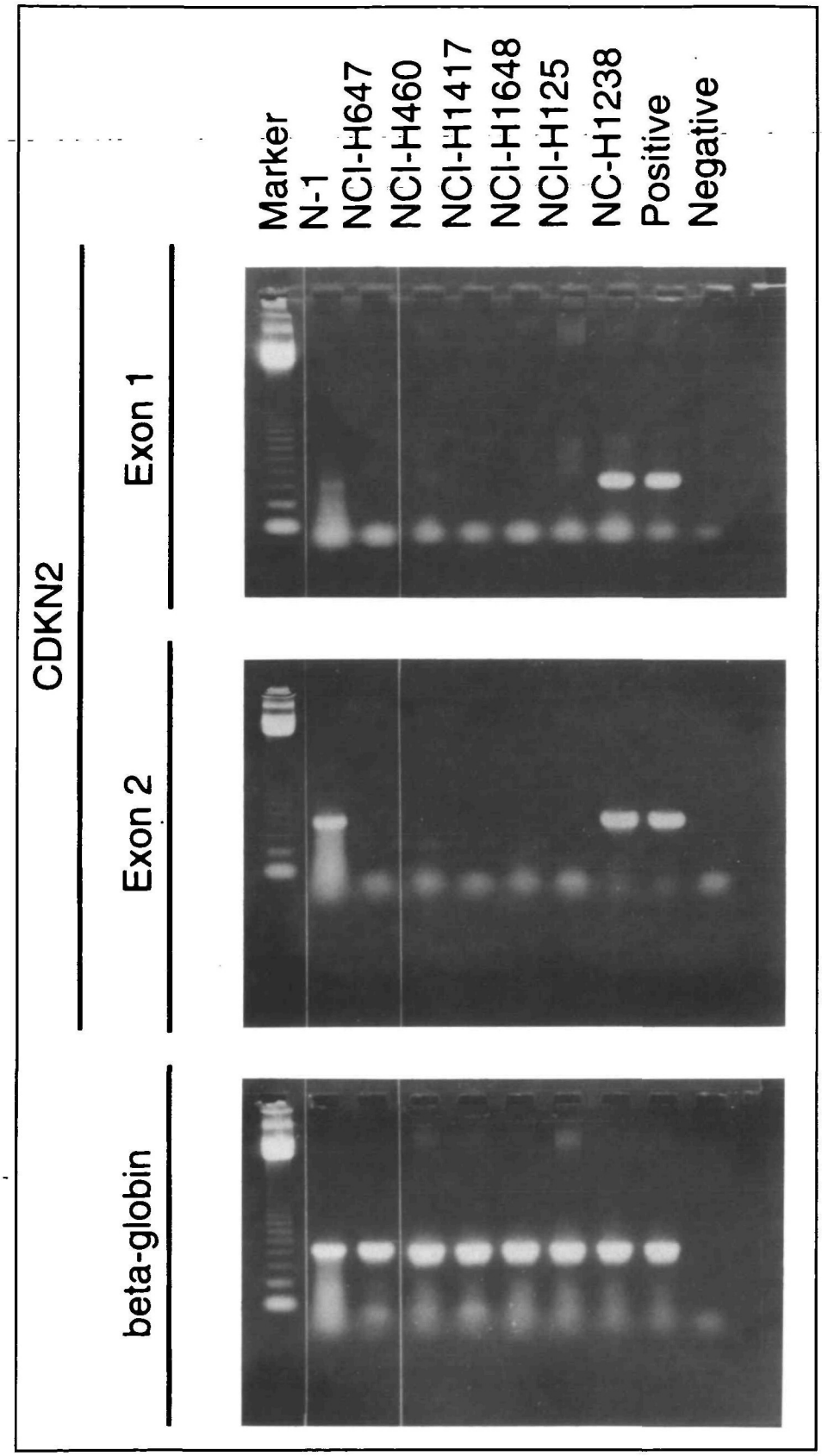

Fig. 1. Homozygous deletions of CDKN2 in lung cancer cell lines. CDKN2 exon 1 (upper panel), exon 2 (middle panel), and a beta-globin gene segment (lower panel) were amplified from the DNA of nonmalignant cells (N-1) and the corresponding tumor cell line from a patient with adenocarcinoma (NCI-H647). Lung cancer cell lines studied without the corresponding normal tissue included NCI-H460 (large-cell carcinoma), NCI-H1417 (SCLC), NCI-H1648 (adenocarcinoma), NCI-H125 (squamous cell carcinoma), and NCI-H1238 (SCLC). Amplification reactions using normal human lung DNA isolated from a patient without cancer (positive) and with no added DNA (negative) were also evaluated.

analysis of the CDKN2 gene in 22 primary cancers that were surgically isolated in the United States (including 15 known to have lost 9p alleles or a single chromosome 9) showed no mutations in the CDKN2 coding region (*35,36*). In contrast, 19 (30%) of 64 primary NSCLCs from Japan were found to have somatic mutations (*37*). Potential explanations for these findings include the following: 1) The tumors evaluated in the two countries were obtained from dissimilar stages or histologies (none of the studies give stage, and the U.S. studies do not state histology); 2) there were differences in technical factors, such as sequencing errors; or 3) the mechanisms or frequency of inactivation of the CDKN2 gene differed in the two populations. Additional research is needed to determine whether deletions and mutations of the CDKN2 gene arise late in the developmental stages of lung cancer or whether this represents an artifact.

Homozygous deletion of the CDKN2 gene are present in 25% of the NSCLC cell lines studied thus far [this study; (*38,39*)]. In contrast, homozygous deletions of DNA fragments from chromosome 3p (*38-40*), the TP53 gene (*41-45*), and the RB gene (*13,15,17*) are detected in less than 5% of tumor cell lines studied. Therefore, the observation of CDKN2 deletion in 25% of NSCLC cell lines makes homozygous deletions in this gene more common than in the other previously characterized tumor suppressor genes and candidate tumor suppressor genes associated with lung cancer.

Homozygous deletion of the CDKN2 gene in 25% of NSCLC cell lines identifies another molecular lesion in the control of the cell cycle in lung cancer cell lines. This is the minimal estimate of the inactivation of the CDKN2 gene in NSCLC cell lines, as small insertions, deletions, and point mutations would not be detected by our method of analysis. In the current study, the inability to detect CDKN2 mRNA expression in some lung cancer cell lines that retain exons 1 and 2 (representing 97% of the coding region) of the CDKN2 gene and the failure to detect the encoded p16 polypeptide in 23 of 33 (70%) of NSCLC cell lines (*46*) raise the possibility of genetic alteration of the CDKN2 promoter. However, these observations may reflect an epigenetic

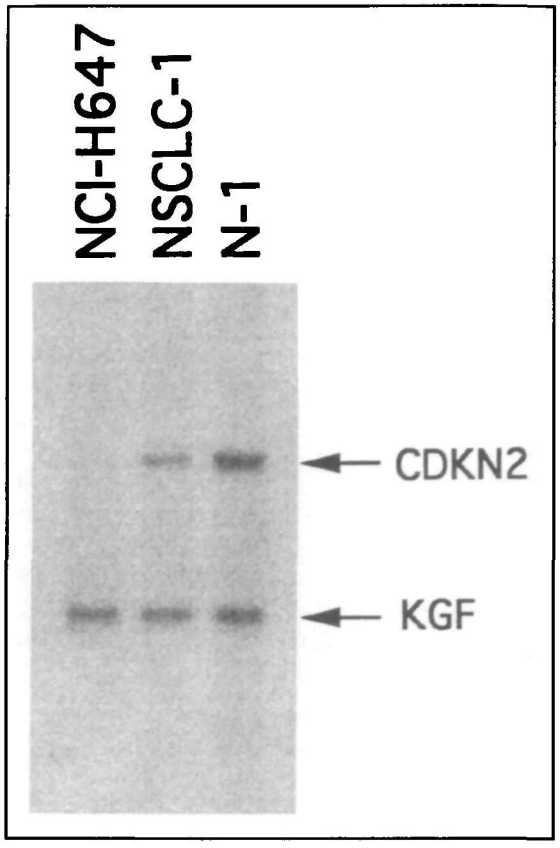

Fig. 2. Southern blot analysis of CDKN2 in a lung cancer cell line, the corresponding tumor, and normal tissues. *Eco*RI-digested genomic DNA isolated from the NSCLC cell line NCI-H647, the primary tumor tissue (NSCLC-1), and the normal tissue (N-1) of the same patient was subjected to Southern blot analysis as previously described (*29*) with a radiolabeled genomic CDKN2 exon 1 fragment probe. A fragment of approximately 4 kb in size was detected in the tumor and normal tissue DNA (upper arrow), while the control KGF exon 1 probe identified a 3.5-kb fragment in each DNA sample (lower arrow). The relative intensity of the CDKN2 exon 1 signal in the tumor tissue was compared with that obtained from the normal tissue, after correcting for differences in the control KGF signal intensities.

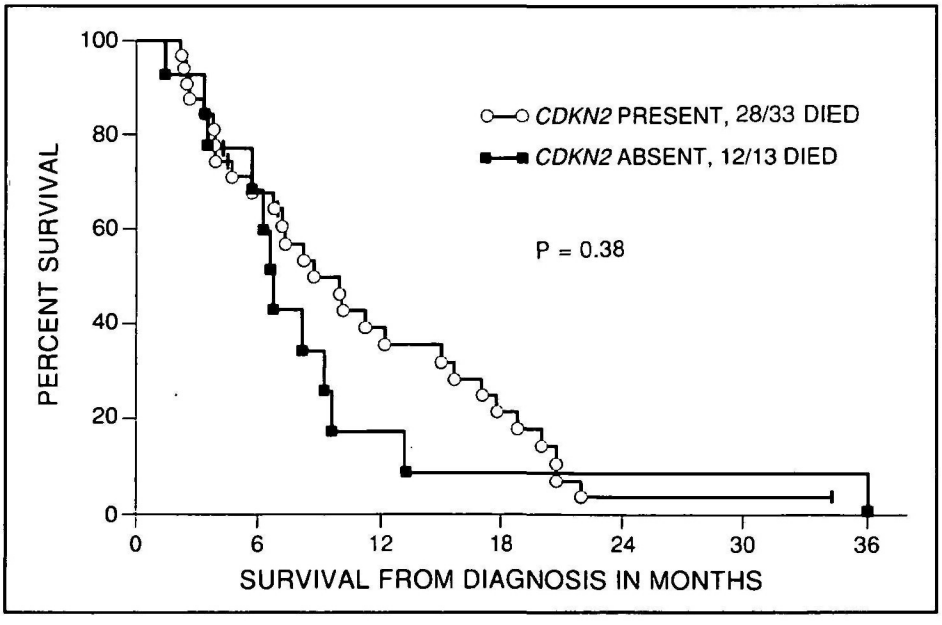

Fig. 3. Survival analysis of CDKN2 homozygous deletions in patients with stage III or IV NSCLC. Survival in months is plotted for the 33 patients with stage III or IV NSCLC whose cell lines did not have homozygous deletion of CDKN2 (○) and the 13 patients with homozygous deletion of CDKN2 (■) by the method of Kaplan–Meier.

phenomenon, such as the recently demonstrated suppression of CDKN2 expression by normal Rb protein (*47*).

The more frequent occurrence of CDKN2 homozygous deletions in NSCLC than SCLC initially suggested that abnormalities of the CDKN2 gene might occur only in tumor cells with normal Rb protein. Such a model would imply that only one defect in the control of the G_1-S cell cycle restriction point would be required for the transformation of a normal cell to a malignant cell. However, our finding of coincident loss of the CDKN2 gene and Rb protein in two lung cancer cell lines demonstrates that, during carcinogenesis, CDKN2 and Rb inactivation are not invariably mutually exclusive events. While this may result from inherent genomic instability in these tumor cells, it is also possible that the functions of the CDKN2 gene product and the Rb protein are not exclusively codependent or that there is another tumor suppressor gene near CDKN2 that is a target of these deletions.

Research is ongoing to attempt to identify additional molecular lesions in the CDKN2 gene and its encoded protein. The ultimate role of the CDKN2 gene product as a potential suppressor of lung cancer development awaits nucleotide sequence analysis of the remaining CDKN2 alleles in lung cancer cell lines with hemizygous deletions of the gene and further analysis of primary tumor samples.

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Notes

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