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A Comprehensive Analysis of p16 Expression, Gene Status, and Promoter Hypermethylation In Surgically Resected Non-small Cell Lung Carcinomas

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Introduction: The role of p16 is gaining importance in non-small cell lung cancer (NSCLC) because of epigenetic therapy options. Further insight into the significance of protein expression, gene status and promoter methylation is needed and has the potential to optimize existing treatment strategies.

Methods: This population-based study analyzes p16 in 383 surgically resected non-small cell lung carcinomas brought into a standardized tissue microarray platform. Immunohistochemistry and fluorescence in situ hybridization were performed. For selected cases, p16 promoter hypermethylation was assessed by a pyrosequencing assay. Extensive clinical data and a postoperative follow-up period of 15 years enabled detailed correlations.

Results: Loss of p16 expression is a common event in NSCLC (232/365, 64%), especially in squamous cell carcinomas (97/115, 84%) in contrast to adenocarcinomas (93/186, 50%). Loss of p16 expression was associated with poorer survival time for the entire cohort and for certain subgroups including men, age younger than 65 years, smokers, early tumor stage, adenocarcinoma, and large-cell carcinoma. Promoter hypermethylation was absent for cases expressing p16 but was commonly observed when (heterozygous) *p16* gene deletions were present and in cases negative for p16.

Conclusion: Our comprehensive data would be compatible with a two-step process leading to loss of p16 expression in NSCLC. Hypermethylation of the promoter region may represent an early event, followed by heterozygous deletion of the *p16* locus. Because of the possibility of detection of hypermethylated gene regions, these data may lead to the identification of specific patient subgroups more likely to benefit from upcoming demethylating treatment strategies.

Key Words: p16, Expression, Deletion, Hypermethylation, Non-small cell lung cancer.

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The *p16* gene, a well-known tumor suppressor gene, is localized on chromosome 9p21 and its product inhibits cyclin D-dependent phosphorylation of the retinoblastoma protein by binding the cyclin-dependent kinases (cdks), cdk4 and cdk6. Loss of p16 leads to phosphorylation of the retinoblastoma protein, releasing cell cycle inhibition and allowing uncontrolled progress from the G1- to the S-phase. Loss of p16 has been described for a variety of tumors including non-small cell lung cancer (NSCLC) and is usually associated with worse prognosis.[1] Major mechanisms of decreased activity of p16 include gene deletion and hypermethylation of the cytosine-phosphate diester-guanine island promoter region, both being observed in NSCLC.[2,3] DNA methylation plays an essential role in the maintenance of genomic stability; however, alterations in methylation patterns frequently occur in tumor cells.[4] Hypermethylation in the promoter regions of tumor suppressor genes is commonly associated with epigenetically mediated gene silencing.[5] In lung cancer, *p16* gene hypermethylation has been detected in 17 to 84% of cases in a smoking habit-dependent manner and may be a candidate marker for predicting the prognosis of NSCLC.[6,7] Gene promoter hypermethylation in sputum cells has even been reported as a molecular marker for identifying healthy individuals at high risk for cancer incidence.[8] Epigenetic changes involved in cancer development, unlike genetic changes, are reversible. Thus, patients with NSCLC with epigenetic alterations could benefit from treatment with demethylating agents after surgery. Azacytidine is the first DNA hypomethylating agent approved by the Food and Drug Administration for the treatment of myelodysplastic syndromes with demonstrated efficacy, and prospective studies in NSCLC with promising results are ongoing.[9,10] So far, predictive markers for response to demethylating agents have not been established.

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To date, various detailed reports on p16 in NSCLC are mainly concentrated on protein expression analysis by immunohistochemistry.[11–15] Studies concerning gene status and hypermethylation of the promoter region are usually limited to general findings without correlation with protein expression.[2,6,7] We aimed to analyze a large group of surgically resected NSCLC regarding p16 protein expression, *p16* gene copy number status, and for selected cases p16 gene promoter hypermethylation. The relationship between protein expression, gene status, and promoter hypermethylation was examined and correlated with extensive clinical data over a follow-up period of up to 15 years after surgical tumor resection.

MATERIALS AND METHODS

Patients and Tissue Sampling

The archival samples were derived from 383 patients with NSCLC with radical surgical resection in curative intent between 1992 and 2004 and diagnosed at the Institute for Pathology, Medical University of Innsbruck. Cases were selected only based on tissue preservation. Hematoxylin and eosin (HE)-stained slides from all available specimens were reclassified by two pathologists (W.S. and A.T.) without knowledge of patient's data, according to the current World Health Organization classification of tumors of the lung as described previously.[16–18] Categories included squamous cell carcinoma (SCC), adenocarcinoma (ACA), large-cell carcinoma (LCC), adenosquamous carcinoma, sarcomatoid carcinoma (which were all of the pleomorphic type), and mucoepidermoid carcinoma. Carcinoids were excluded from this analysis. Tumor differentiation was graded as well, moderate, or poor. The clinical information was documented within the Twelve Years Retrospective of Lung Cancer survey, a project aiming to analyze various features of a large number of patients with lung cancer.[19] These patients mainly originated from the Austrian province of Tyrol and were all treated at the Medical University Hospital of Innsbruck and in associated hospitals. Approval for data acquisition and analysis was obtained from the local Institutional Review Board, i.e., the Ethics Committee of the Medical University of Innsbruck. The basic patients' characteristics including symptoms at presentation, smoking habits, comorbidities, and laboratory parameters, as well as the complete course of treatment modalities, including surgery and all lines of chemotherapy and radiotherapy and continuous follow-up and end point data, were documented.[19] Regarding therapy modalities, the patients were routinely discussed at the Medical University of Innsbruck Tumor Board, and a state of the art recommendation of therapy, adapted to the condition of the patient, was given. Accordingly, there was a continuous shift of therapy modalities routinely applied in this comparably large interval of study recruitment.[19,20]

Tissue Microarray Construction

Tumor material consisted of paraffin-embedded tissue after fixation in 10% neutral buffered formalin. The tissue microarray (TMA) was constructed as previously described.[17,21] Briefly, representative intratumoral areas were marked on HE-stained slides, and four cylindrical 0.6-mm tissue cores each were arrayed from the corresponding paraffin blocks into a recipient block using an arraying machine from Beecher Instruments (Sun Prairie, WI). The core coordinates were recorded for exact location using Microsoft Excel (Microsoft, Redmond, WA) and printouts assisted the subsequent immunohistochemical evaluation. Four-micrometer-thick paraffin sections were cut. The first section was stained by HE to confirm validity, the rest were used for immunohistochemistry. Adhesive transfer tape was not used.

Immunohistochemistry

For detection of p16, the ready to use kit of CINtec (Heidelberg, Germany) was used. Immunohistochemistry was performed using the automated staining system, Benchmark XT (Roche/Ventana Medical Systems, Tucson, AZ). Antigen retrieval was done by CC1 buffer. Incubation lasted for 32 minutes at 37°C. Diaminobenzidine was used as a chromogen. Stainings for other markers were performed as previously described.[17,21] The percentage of positively stained tumor cells was established by actual cell count independently by two pathologists (W.S. and A.T.) to study interobserver agreement. The percentage of positive tumor cells was noted for each spot, followed by the calculation of the arithmetic mean value. The prognostic relevance and the cutoff score to determine positivity were assessed by means of receiver operating characteristic (ROC) analysis, selecting death as the state variable (see Statistical Analysis).[22]

Fluorescence In Situ Hybridization

To study the *p16* gene status, interphase fluorescence in situ hybridization (FISH) with a locus-specific identifier *p16* (*9p21*) SpectrumOrange/chromosome enumeration probe (CEP) 9 SpecturmGreen dual-color mix (05J51-001 from Abbott/Vysis, Baar, Switzerland) was performed on TMA sections according to the manufacturer's protocol. The sections were further processed with a paraffin pretreatment reagent kit (Abbott/Vysis), and hybridization was performed as described in Vysis' protocol. Denaturation lasted for 10 minutes at 73°C, and FISH mix was incubated overnight at 37°C in Hybrite (Abbott/Vysis). Slides were counterstained with 125 ng/ml 4′, 6-diamino-2-phenylindole in antifade solution. FISH signals were visualized on a Zeiss fluorescence microscope equipped with double-band pass filters for simultaneous visualization of green and red signals. Cases were considered evaluable for FISH if at least 100 tumor cell nuclei/core displayed positive signals. Red and green signals were counted separately in 20 cells. The red to green ratio was calculated mathematically. Deletions of *9p21* were defined as red to green ratio below the mean minus 3 SDs of red to green ratios in reference cases (normal lung parenchyma, $n = 5$), i.e., a ratio of less than 0.636. This cutoff score also best discriminated between immunohistochemically p16-positive and -negative cases with a specificity of 60% and a sensitivity of 45% as determined by ROC (see below), with an area under the ROC of 0.601 (95% CI: 0.545–0.675, $p = 0.001$).

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p16 Pyrosequencing for the Detection of p16 Promoter Hypermethylation

Genomic DNA was extracted from paraffin-embedded tumor tissue using the QIAamp DNA Mini Kit (Cat. No 51304, Qiagen, Valencia, CA). Universal Methylated Human DNA Standard (Cat. No. D5011, Zymo Research Corp., Irvine, CA) was used as DNA methylation-positive control and DNA from normal human lymphocytes as methylation-negative control. Bisulfite conversion of the DNA was performed using the EZ DNA Methylation-Gold Kit (Cat. No. D5006, Zymo Research Corp.) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) amplification of bisulfite-converted DNA was performed using specific *p16* PCR primers resulting in a PCR product including five CpG sites. For the first and the nested PCR, 40 amplification cycles were performed. Two microliters of DNA was added in a volume of 25 μl containing dNTPs (0.2 mM final), PCR Gold Buffer (1× final; Applied Biosystem), PCR primers (0.12 μM), and 0.2 μl of *Taq* polymerase (ampliTaq Gold; Applied Biosystem). The nested PCR was performed under the same condition by adding 1 μl of the amplicon in 25 μl of master mix.

Fifteen microliters of the biotinylated PCR product were immobilized on streptavidin-coated sepharose beads by mixing at 1400 rpm at a room temperature for 5 minutes. Then beads with immobilized PCR product were picked up by Vacuum Prep Tool from the PCR plate and moved to a separate one. In this study, 70% ethanol is aspirated through the filter probes and placed in a trough of sodium hydroxide to denature the DNA (biot. DNA strand remains immobilized on the beads). The tool is placed in a trough of washing buffer and rinsed by aspiration. The single-stranded templates are transferred to a prepared plate containing annealing buffer and sequencing primer (25 μl). Release of the beads takes place with the template into the plate by switching off the vacuum pressure. After that the annealing of primer can be started at 80°C for 2 minutes, and the samples are ready for analysis.

Pyrosequencing was subsequently carried out in the PyroMark Q24 instrument (Qiagen). The degree of methylation of all five CpG sites was automatically analyzed by the PyroMark Q24 software. A cutoff of 10% methylated DNA content was defined as positive for *p16* promoter hypermethylation. Primer sequences for *CDKN2A* first round PCR were ATGGAGTTTTYGGTTGATTGGT (forward) and CCCCCC-ATCCCCTACTCC (reverse). Primer sequences for *CDKN2A* nested PCR were ATGGAGTTTTYGGTTGATTGGT (forward), GGAGTTTTAGGTTGATTGGGTT (reverse), and biot-CCCTCTACCCACCTAAAT (sequencing primer).

Statistical Analysis

The degree of agreement between observers was evaluated by interclass correlation coefficients, using reliability Cronbach's alpha analysis. Correlation analysis of clinicopathological and immunohistochemical parameters was performed using the Spearman test; after correction for multiple testing (0.05 divided by the number of analyzed parameters), p values ≤ 0.008 were considered as significant. In addition, for the three major histology types (ACA, SCC, and LCC), the mean percentage of positively stained cells was compared, and significant markers (p value$^{ANOVA} < 0.005$) were further analyzed using the Kruskal-Wallis H test. The discriminatory power of red to green FISH ratios to predict immunohistochemical detectability of p16 was assessed by means of ROC analysis, selecting expression of p16 as the state variable. Optimal cutoff values were calculated using the Youden index (J) for maximum of specificity and sensitivity.[22] The prognostic relevance of respective markers was assessed also by means of ROC analysis, selecting death as the state variable. Optimal cutoff values were calculated using J for variables demonstrating an asymptomatic significance by ROC below 0.2 and an adequately shaped ROC curve, otherwise the median was selected. Kaplan-Meier curves were calculated for survival estimates and a log-rank statistics used to determine differences between groups; multivariable analysis was performed using the Cox regression model. p -values less than 0.05 were considered as significant. Two-sided tests were used throughout. Statistical calculations were performed using SPSS 19.0 software (SPSS, Chicago, IL).

RESULTS

Histopathology and Patient Characteristics

Histological subtypes consisted of 193 ACAs, 123 SCCs, 54 LCCs, 8 adenosquamous carcinomas, 4 pleomorphic carcinomas, and 1 mucoepidermoid carcinoma. Twenty were well differentiated, 161 moderately differentiated, and 202 poorly differentiated. Distribution of pathological tumor stage by the Union Internationale Contre le Cancer (UICC) was as follows: 103 stage IA, 113 stage IB, 19 stage IIA, 48 stage IIB, 63 stage IIIA, 18 stage IIIB, and 19 stage IV. The patient population comprised 280 men and 103 women (ratio 2.7:1) with an average age of 63 years and a range from 23 to 84 years. For 193 of 383 (50.4%) patients, the smoking history was known: 13 of 193 (6.7%) were never smokers, 145 of 193 (75.2%) were smokers (either current or quit less than 2 years before diagnosis), and 35 of 193 (18.1%) were former smokers (quit longer than 2 years before diagnosis).

Immunohistochemistry

Three hundred sixty-five cases qualified for evaluation of immunohistochemistry. Eighteen cases were excluded due to lack of tumor cells (<20). By ROC analysis, p16 discrimination power for overall survival time after radical surgery resulted in: area under the ROC = 0.537, 95% CI: 0.478–0.598, and $p = 0.2$. The calculated cutoff level (J) was 10%. In all, 232 of 365 (63.6%) cases expressed p16 below the cutoff level (loss of p16 expression), and 133 of 365 (36.4%) cases were considered p16-positive. Cronbach's alpha for interobserver reproducibility of the immunohistochemistry was excellent ($\alpha = 0.987$). Most positive cases showed a diffuse and strong staining pattern of all the tumor cells (Figure 1*A*). Staining was localized to the cytoplasm in 84 cases, to the nucleus in two cases, and both nucleus and cytoplasm were positively stained in 47 cases. Endothelial

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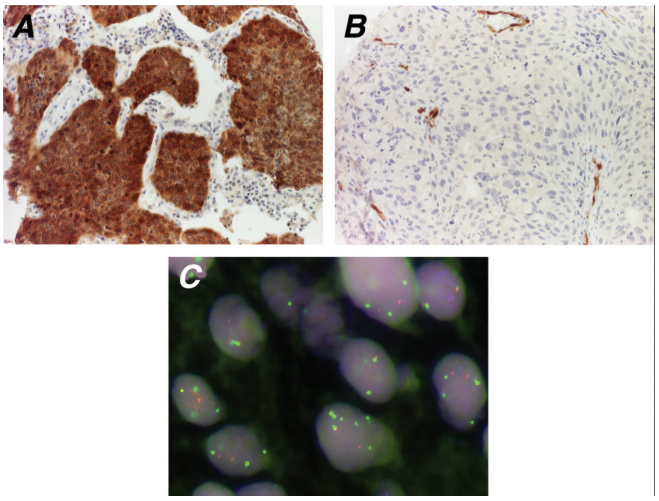

FIGURE 1. Immunohistochemistry for p16 demonstrating a diffusely positive squamous cell carcinoma (*A*) and a negative adenocarcinoma with positively stained interspersed endothelial and mesenchymal cells (*B*). Original magnification ×40. (*C*) Fluorescence in situ hybridization analysis with a locus-specific identifier (LSI) *p16 (9p21)* SpectrumOrange/chromosome enumeration probe (CEP) 9 SpecturmGreen dual-color mix. Note increased numbers ($n = 30$) of green (C*EP9*) compared with red ($n = 17$) signals (9p21) (red/green ratio 0.567) corresponding to p16 deletion.

and mesenchymal cells served as a positive internal control for negative tumors (Figure 1*B*). The mean percentage of stained tumor cells for the entire cohort was 23.4% (range 0–100; median: 0). Mean percentage of stained tumor cells according to main histological subtype was 12.8% for SCC, 29% for ACA, and 31.5% for LCC ($p < 0.001$). Loss of p16 expression was frequently found in SCC (97/115, 84%) in contrast to ACA (93/186, 50%). For distribution of cases according to the main histological subtypes, see Table 1. The cyclin D1 and epidermal growth factor receptor (EGFR)-staining results have been described in detail earlier in the text.[21]

Fluorescence In Situ Hybridization

Three hundred twenty-two cases were evaluable for FISH. Problems related to the tissue preservation with weak or lacking FISH signals were responsible for the majority of noninformative cases ($n = 46$), whereas problems related to the TMA technology such as lacking punches accounted for the minority of noninformative cases ($n = 15$). The mean red (9p21)/green (CEP9) ratio was 0.887, median 0.938, and range 0.340–1.143. Altogether, 34 cases (10.4%) were considered to harbor 9p21 (*p16*) deletions with a mean red (9p21)/green (CEP9) ratio of 0.502, median 0.515, and range 0.340–0.633 (Figure 1*C*). The nondeleted cases had a mean red (9p21)/green (CEP9) ratio of 0.932, median 0.943, and range 0.636–1.143 (p for difference between both groups <0.001). There were no NSCLC with homozygous p16 deletion. Because of slight nonoverlap of noninformative cases for FISH and immunohistochemistry, in altogether 317 cases there was information on both deletion status and protein expression (Figure 2).

p16 Pyrosequencing

To test the influence of 9p21 deletions and *p16* promoter hypermethylations on the p16 protein expression status, we selected, based on best tissue preservation and taking into account representation of the three major histological subtypes (ACA, SCC, and LCC), nine nondeleted and p16-negative cases, five nondeleted and p16-postive cases, nine deleted and p16-negative cases, and five deleted and p16-positive cases for methylation analysis by pyrosequencing. Results are shown in Figure 2. Importantly, *p16* promoter hypermethylation was observable only in p16-negative cases (6/18), whereas no hypermethylation was found among p16-positive cases (0/9). *p16* promoter hypermethylation was twice as frequent in *p16*-deleted cases (4/9) compared with nondeleted cases (2/9).

Correlations Between Variables

Loss of expression of p16 was associated with SCC histology (Spearman correlation coefficient: $\rho = 0.308$; $p < 0.001$), earlier tumor stage ($\rho = 0.162$; $p = 0.002$), and increased expression of EGFR ($\rho = 0.190$; $p < 0.001$) and cyclin D1 ($\rho = 0.140$, $p = 0.008$). In contrast, p16 expression was frequently detected in ACA ($\rho = 0.286$; $p < 0.001$). A lower ratio of 9p21/CEP9 (*p16* gene deletion) correlated with loss of p16 protein expression (ρ: 0.173; $p = 0.002$) and with higher tumor grade (ρ: 0.152; $p = 0.006$). Case distribution of p16 protein expression and gene status among major histological subtypes is shown in Table 2, and distribution of p16 protein expression and promoter hypermethylation among major histological subtypes is shown in Table 3. Smoking status did not correlate with p16 expression or gene status. The combination of *p16* gene deletion and promoter hypermethylation was not associated with other parameters, although the small number of such cases ($n = 4$) does not allow precise conclusions.

Survival Analysis

The median follow-up time was 41.8 months, the longest being 181.1 months. Results for calculations of survival estimates for the entire group are shown in Table 1. Loss of p16 expression was associated with significantly worse overall survival for the whole cohort (Figure 3*A*). Within subgroups, this applied for patients aged 55 years or younger, male gender (Figure 3*B*), smokers, pUICC stage IA and IB, pN stage 0, pM stage 0, well or poor (not for moderate) tumor differentiation, nonrecurring tumors, and for ACA (Figure 3*C*) and LCC histology. When patients were grouped as either 65 years or younger or older than 65 years of age, loss of p16 expression was also associated with significantly worse overall survival for patients who were 65 years or younger: 125 of 207 (60.4%) p16-negative patients had a median overall postoperative survival time of 45.5 months, compared with 84.5 months for 82 of 207 (39.6%) p16-positive patients ($p = 0.002$) (Figure 3*D*). Combined analysis of p16 and cyclin D1 showed that cases with loss of p16 with concurrent overexpression of cyclin D1 (cutoff = 15% pos-

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TABLE 1. Postoperative OS Analysis by Kaplan-Meier Estimates According to Clinicopathological Parameters and p16 Expression

	p16 Expression <10%, No. of Patients (%)	Median OS in mo Above (SD) Median RFS in mo Below (SD)	p16 Expression ≥10%, No. of Patients (%)	Median OS in mo Above (SD) Median RFS in mo Below (SD)	*p*
Parameter					
All	232/365 (63.6)	40.8 (4.6)	133/365 (36.4)	67.2 (14.4)	0.002
		51.8 (21.8)		115.3 (—)	0.101
Gender					
Female	58/98 (59.2)	62.1 (28.8)	40/98 (40.8)	94.5 (25.4)	0.107
		76.7 (32.6)		NR	0.053
Male	174/267 (65.2)	35.7 (4.4)	93/267 (34.8)	52.8 (10.2)	0.012
		46.0 (23.8)		75.6 (32.3)	0.507
Histology					
ACA	93/186 (50)	38.9 (6)	93/186 (50)	72.5 (14.8)	0.020
		42.0 (13.6)		93.2 (—)	0.117
SCC	97/115 (84.3)	51.5 (12)	18/115 (15.7)	49.8 (18.2)	0.463
		NR		NR	0.316
LCC	32/52 (61.5)	16.8 (8.9)	20/52 (38.5)	89.9 (39.2)	0.010
		49.5 (23.7)		94.2 (55.0)	0.382
Other	10/12 (83.3)	18.9 (5.5)	2/12 (16.7)	59.7 (—)	0.395
		8.0 (2.3)		28.4 (—)	0.402
Differentiation					
Well	9/20 (45)	17.2 (36.1)	11/20 (55)	131.3 (46)	0.023
		76.7 (22.5)		NR	0.266
Moderate	98/155 (63.2)	54 (15.1)	57/155 (36.8)	61.2 (15.6)	0.219
		65.7 (28.1)		86.5 (—)	0.802
Poor	125/190 (65.8)	36.3 (4.6)	65/190 (34.2)	49.8 (17.1)	0.029
		42.0 (16.1)		115.3 (—)	0.153
Smoking status					
Never smoker	6/11 (54.5)	47.5 (39.4)	5/11 (45.5)	76.2 (12.2)	0.643
		76.7 (42.9)		73.9 (42.4)	0.852
Smoker	87/137 (63.5)	38.5 (7)	50/137 (36.5)	59.7 (13.7)	0.019
		42.0 (12.3)		115.3 (—)	0.120
Former smoker	28/35 (80)	92.3 (18.7)	7/35 (20)	89.8 (25)	0.833
		104.7 (51.9)		NR	0.881
pUICC					
IA	51/97 (52.6)	47.5 (7.9)	46/97 (47.4)	118 (20.5)	0.011
		61.2 (—)		NR	0.139
IB	79/110 (71.8)	61.6 (12)	31/110 (28.2)	121.2 (—)	0.009
		120.6 (18.8)		NR	0.848
IIA	12/19 (63.2)	33.1 (3.8)	7/19 (36.8)	75.1 (36.3)	0.210
		26.6 (11.8)		NR	0.062
IIB	35/43 (81.4)	40.8 (27.6)	8/43 (18.6)	39.9 (6.9)	0.237
		46.0 (43.1)		14.8 (1.4)	0.239
IIIA	38/62 (61.3)	19.7 (6)	24/62 (38.7)	24 (13.5)	0.323
		14.5 (5.5)		35.2 (59.6)	0.129
IIIB	9/17 (52.9)	13.8 (0.7)	8/17 (47.1)	22.9 (10.4)	0.767
		NR		63.3 (39.7)	0.953
IV	8/17 (47.1)	15.1 (12.1)	9/17 (52.9)	25.2 (0.9)	0.360
		27.8 (10.7)		18.2 (4.8)	0.729

(*Continued*)

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TABLE 1. (*Continued*)

	p16 Expression <10%, No. of Patients (%)	Median OS in mo Above (SD) Median RFS in mo Below (SD)	p16 Expression ≥10%, No. of Patients (%)	Median OS in mo Above (SD) Median RFS in mo Below (SD)	*p*
T stage					
1	68/133 (51.1)	47.5 (6.5)	65/133 (48.9)	84.5 (18.7)	0.023
		49.5 (14.3)		NR	0.105
2	133/186 (71.5)	43.1 (9.9)	53 (28.5)	48.3 (13.4)	0.133
		95.0 (27.8)		44.7 (28.3)	0.957
3	21/29 (72.4)	18.9 (6.8)	7/29 (827.6)	32.8 (—)	0.225
		19.4 (9.4)		NR	0.208
4	10/18 (55.5)	13.8 (7.4)	8/18 (44.5)	22.9 (10.4)	0.765
		17.8 (10.6)		63.2 (39.7)	0.928
N stage					
0	146/235 (62.1)	47.8 (7.5)	89/235 (37.9)	118 (20.3)	<0.001
		104.7 (27.1)		NR	0.133
1	59/80 (73.7)	36.3 (7.4)	21/80 (26.3)	42.2 (6.8)	0.876
		31.4 (19.8)		86.5 (59.5)	0.441
2	27/50 (54)	19.7 (6.2)	23/50 (46)	15.4 (5.7)	0.965
		14.5 (4.9)		12.4 (3.1)	0.495
M stage					
0	224/348 (64.4)	41.8 (5)	124/348 (35.6)	75.1 (11)	0.002
		57.7 (23.6)		115.3 (—)	0.090
1	8/17 (47.1)	15.1 (12.1)	9/17 (52.9)	25.2 (0.9)	0.360
		27.8 (10.7)		18.2 (7.8)	0.729
Recurrence					
No	127/194 (65.5)	92.3 (14)	76/194 (34.5)	173.4 (43.9)	0.005
		—		—	—
Yes	105/162 (64.8)	27.9 (3.1)	57/162 (35.2)	35.8 (4.8)	0.397
		12.8 (2.0)		15.0 (2.1)	0.276
Age (yr)					
≤55	43/78 (55.1)	31.2 (14.9)	35/78 (44.9)	173.4 (61.2)	0.006
		19.2 (3.8)		115.3 (66.8)	0.077
>55 to ≤65	82/129 (63.6)	62.1 (13.4)	47/129 (36.4)	84.1 (25.4)	0.082
		57.7 (30.1)		NR	0.099
>65 to ≤75	79/116 (68.1)	31.6 (4.6)	37/116 (31.9)	52.8 (11.5)	0.212
		76.7 (34.9)		NR	0.672
>75	28/42 (66.6)	40.7 (13.1)	14/42 (33.4)	42.2 (2.2)	0.910
		NR		23.0 (42.1)	0.498

RFS, recurrence-free survival; SD, standard deviation; NR, not reached; OS, overall survival; ACA, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large-cell carcinoma; UICC, Union Internationale Contre le Cancer.

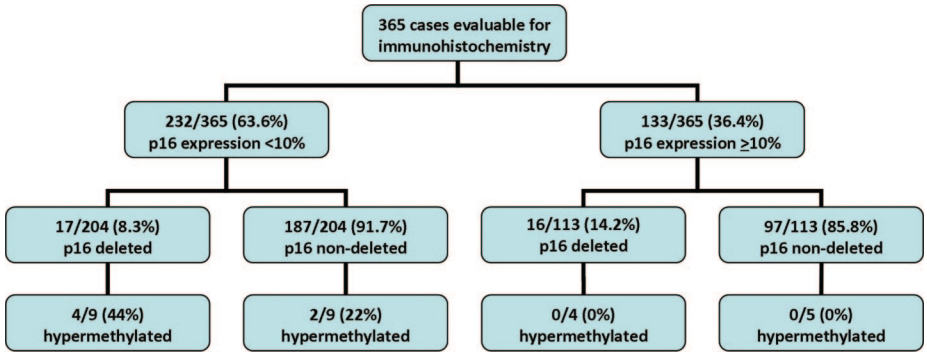

FIGURE 2. Flowchart of the assessment of protein expression, gene status and promoter hypermethylation of p16 in non-small cell lung cancer.

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TABLE 2. Case Distribution of p16 Protein Expression and Gene Status Among Major Histological Subtypes

	SCC (%)	ACA (%)	LCC (%)
p16 expression <10% and gene deletion	10 (9.7)	6 (3.9)	1 (2)
p16 expression ≥10% and no gene deletion	9 (8.7)	73 (47.8)	13 (26)
p16 expression ≥10% and gene deletion	5 (4.9)	6 (3.9)	5 (10)
p16 expression <10% and no gene deletion	79 (76.7)	68 (44.4)	31 (62)
Total	103	153	50

SCC, squamous cell carcinoma; ACA, adenocarcinoma; LCC, large-cell carcinoma.

TABLE 3. Case Distribution of p16 Protein Expression and Promoter Methylation Among Major Histological Subtypes

	SCC (%)	ACA (%)	LCC (%)
p16 expression <10% and hypermethylation	4 (36.4)	2 (18.1)	0
p16 expression ≥10% and no hypermethylation	2 (18.1)	5 (45.5)	3 (60)
p16 expression ≥10% and hypermethylation	0	0	0
p16 expression <10% and no hypermethylation	5 (45.5)	4 (36.4)	2 (40)
Total	11	11	5

SCC, squamous cell carcinoma; ACA, adenocarcinoma; LCC, large-cell carcinoma.

itively stained tumor cells, as previously described) were associated with the worst overall survival time, whereas cases with detectable p16 expression and cyclin D1 levels below the cutoff level demonstrated significantly longer survival (p16+/cylin D1−: 106/363 [29.2%] cases, 76.2 months median; p16+/cyclin D1+: 27/363 [7.4%] cases, 42.2 months median; p16−/cyclin D1−: 162/363 [44.6%] cases, 44.6 months median; and p16−/cyclin D1+: 68/363 [18.8%] cases, 36.9 months median; $p = 0.03$).[21] *p16* gene deletions were associated with poor overall survival for women. Recurrence-free survival (RFS) was defined as the time from operation until disease recurrence, including patients without recurrence who were censored at date of last follow-up or death. Median RFS was 28.7 months. Results for calculations of RFS are listed in Table 1. In this cohort, loss of p16 expression was not associated with significantly decreased RFS time for the analyzed patient groups. The multivariable analysis included all baseline (i.e., recurrence status excluded) parameters with a p value of less than 0.1 by univariable analysis and revealed that only loss of p16 (relative risk: 1.572, 95% CI: 1.026 – 2.410, $p = 0.038$) was significantly associated with decreased overall survival. Concerning gene deletion of *p16*, the only significant result regarding overall survival time in association with clinicopathologic characteristics was shorter survival for female patients with *p16* deletions. In all, 70/79 (88.6%) women had no detectable *p16* gene deletion and a median overall survival period of 83.8 months, compared with 9 of 79 (11.4%) with deletions and a median overall survival of 32.7 months ($p = 0.003$).

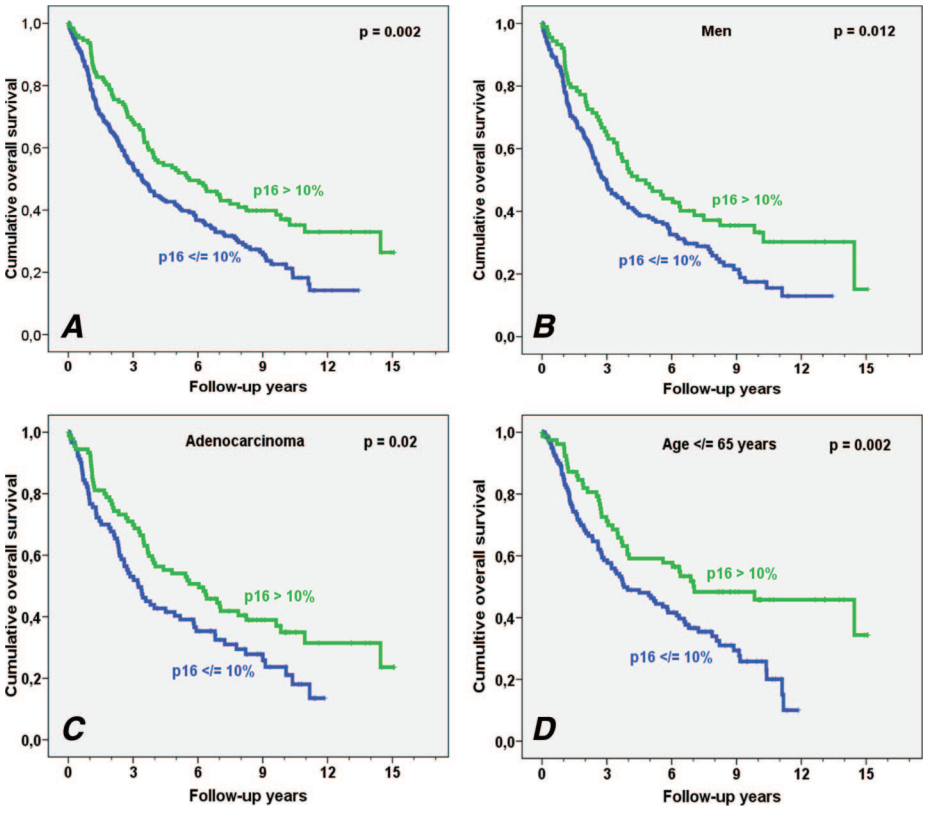

FIGURE 3. Kaplan-Meier estimates of overall survival according to expression of p16 for the entire cohort (*A*), for men (*B*), for adenocarcinomas (*C*), and for patients aged 65 years or younger (*D*).

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DISCUSSION

This population-based study underlines and extends the importance of p16 for surgically resected NSCLC. We found that loss of p16 protein expression is frequent in NSCLC and is an especially common feature of SCC compared with ACA, which is well in line with most smaller previous studies.[11,23] Loss of p16 expression was more frequently observed in earlier tumor stages, although because this is a study of surgically resected NSCLC, there is a natural bias considering tumor stage with proportionally fewer advanced tumors. Expression of p16 and cyclin D1 showed an inverse correlation, which has also been reported by Myong[23] and reflects their functions in the cell cycle. Because p16 inhibits cdks, which become active when bound to cyclin D1, loss of p16 protein can lead to elevated levels of cyclin D1 protein. In our cohort, cases positive for EGFR as assessed by immunohistochemistry more commonly showed loss of p16 expression. This further emphasizes the importance of deregulation of the cell cycle in NSCLC, because activation of EGFR leads to stimulation of the cell cycle whereas p16 is a negative regulator. Such inverse correlations are likely to both stimulate the cell cycle and diminish its inhibition at the same time. Jin et al.[12] stressed that the combination of cyclin D1 expression with loss of p16 expression is very useful to predict the prognosis of patients with NSCLC after curative resection. Indeed, in our collective, the combination of p16 expression loss with cyclin D1 overexpression was associated with reduced overall survival time compared with p16-expression-negative cases with low levels of cyclin D1 expression. For the entire cohort, loss of p16 expression was associated with poor survival, which has previously been widely reported.[13,23] Subgroup analysis revealed that this was only the case for younger patients, male gender, smokers, early tumor stages (pUICC IA and IB), well or poor (not for moderate) tumor differentiation, nonrecurring disease, and for ACA and LCC histology. Few studies have commented on survival with regard to histology, resulting in no association and in divergent findings.[14,24] Notably, to the best of our knowledge, no studies have linked poor survival and loss of p16 expression to age, whereas our data show significantly shorter overall survival time for patients who are 65 years or younger. p16 expression proved to be an independent prognostic factor by multivariable analysis, in line with other reports analyzing surgically resected NSCLC by immunohistochemistry.[13,15] Loss of p16 protein expression by immunohistochemistry is known to be an accurate method for detection of *p16* gene inactivation events.[25] Distinct mechanisms include gene deletions and promoter hypermethylation, whereas point mutations are rare.[25] Although such mechanisms have been described in lung cancer, they have not been assessed simultaneously in a single large tumor series with incorporation of p16 protein expression. Especially the timing of such occurrences is unclear. As expected, a decreased ratio of 9p21/CEP9 correlated with loss of p16 protein expression and was also more common in higher tumor grades. Our data demonstrate that *p16* gene deletions occur at a similar frequency in NSCLC whether p16 protein expression was below or above the cutoff level of 10% (deletion frequency: 8.3% for cases below and 14.2% for cases above the cutoff level). This implies that loss of protein expression is not solely caused by a gene deletion. A likely explanation for the expression of p16 protein despite *p16* deletion is heterozygous deletion. Homozygous deletions on the other hand should be sufficient for loss of protein. In our collective, the 9p21/CEP9 ratio was assessed by FISH, and a cutoff value of 0.636 was established for determining cases harboring a deletion. No tumor had a ratio less than 0.340, thus virtually excluding the possibility of a biallelic deletion. This is in contrast to the study by Gazzeri et al.,[25] who reported frequent homozygote deletions, although they analyzed touch preparations, which is not comparable to our method of interphase FISH on TMA slides.

Given that most SCC were p16-negative, the SCC group of p16-positive cases with nondeleted *p16* gene is especially interesting. Because there may be a connection with human papillomavirus (HPV) infection (e.g., as known for cervical cancer) in SCC expressing p16, we analyzed this group separately regarding a possible viral association. Interestingly, viral infection has also been reported to correlate with better differentiation in SCC of the lung.[26] This small group consisted of nine cases (8.7% of all SCC). Notably, eight patients were men, and the median overall survival time was 73 months compared with 44.7 months for the entire SCC cohort. Tumor stage and grade, Ki67 index, and smoking status did not differ considerably (results not shown). In addition, we performed immunohistochemistry using the Cytoactiv HPV L1 Screening Set (detection of HPV L1 capsid protein) and the Cytoactiv HPV L1 High Risk Set (detection of L1 capsid protein of the high risk HPV subtypes: 16, 18, 31, 33, 35, 39, 45, 56, and 58) (both from Cytoimmun Diagnostics GmbH, Pirmasens/Germany). No case showed a positive nuclear staining signal, thus as expected prognosis was indeed better, although no indication of HPV infection was apparent.

Our cases for gene promoter methylation analysis were selected on grounds to best represent the different groups regarding p16 expression and *p16* gene status (Figure 2). From the analyzed group of tumors with loss of p16 expression, nine *p16* deleted and nine *p16* nondeleted cases were selected. Considering cases with p16 expression, four evaluable of five analyzed cases had deleted and five had nondeleted genes. None of the cases with p16 expression $\geq$10% showed hypermethylated promoter regions (independent of deletion status). However, tumors with loss of p16 expression more commonly harbored *p16* promoter hypermethylation as well, which was twice as frequent for cases with *p16* gene deletions (4/9) compared with *p16* nondeleted instances (2/9). This suggests that hypermethylation of the promoter region of *p16* is most likely the initial step toward loss of p16 function in NSCLC, followed by heterozygous deletion, which finally results in complete loss of protein expression. Studies have demonstrated that aberrant *p16* promoter hypermethylation is an early and critical event in the development of NSCLC, supporting our assumption.[27] Such *p16* promoter hypermethylation has even been observed in serum and sputum of chronic smokers without clinical disease.[8] Analogously to the

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more frequently observed losses of p16 expression in SCC, *p16* promoter hypermethylations have also been more frequently detected in SCC compared with ACA.[7] For cases with loss of p16 expression and no detectable deletion or hypermethylation, the possibility of mutations, and yet undetermined mechanisms of genetic or epigenetic inactivations must be considered.

The importance of the mechanism of gene promoter hypermethylation lies in the possibility of epigenetic treatment options. 5-Azacytidine is a demethylating agent, which inhibits DNA methyltransferase-1 in replicating cells, and 5-aza-deoxycytidine triphosphate (decitabine) is a resulting 5-azacytidine by-product with an additional cytotoxic effect through its incorporation into RNA.[28] Recently, azacytidine has been shown to prolong survival and improve quality of life in patients with myelodysplastic syndromes, while maintaining a favorable adverse effect profile.[9] A recent interim analysis of a phase II trial has reported that the combination of azacytidine and entinostat (inhibitor of histone deacetylase) has a durable benefit in patients with advanced relapsed NSCLC.[10] Pharmacodynamic and pharmacokinetic analyses are being conducted to identify characteristics of the subset of patients responding to this novel therapy.

In conclusion, our data would be compatible with a two-step process leading to loss of p16 protein expression in NSCLC. Hypermethylation of the promoter region of *p16* may present the first event, followed by heterozygote deletion of the *p16* gene locus. We also confirm that loss of p16 expression is a frequent event in NSCLC and is associated with poor survival especially for certain patient subgroups. Considering the possibility of detection of hypermethylated gene regions, these data may lead to the identification of patient subgroups more likely to benefit from upcoming epigenetic treatment strategies.

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