

Role of cdk4, p16^{INK4}, and Rb Expression in the Prognosis of Bronchioloalveolar Carcinomas

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Key Words

p16^{INK4} · Cyclin-dependent kinase 4 · Retinoblastoma protein · Bronchioloalveolar carcinoma

Abstract

Background: The p16^{INK4} protein has been identified as a potent inhibitor of cyclin-dependent kinase (cdk)4 by blocking cdk4-mediated phosphorylation of the tumor suppressor retinoblastoma (Rb) protein, thus allowing Rb-mediated growth suppression. **Objectives:** Loss of p16^{INK4} has been associated with a poor cancer prognosis, but its potential significance in bronchioloalveolar carcinomas (BACs) has not been explored. **Methods:** We examined immunohistochemical expression of p16^{INK4}, cdk4, and Rb proteins in 38 BACs and correlated their expression levels with known clinicopathological features of the disease. **Results:** All BACs expressed cdk4, while 89 and 82% expressed p16^{INK4} and Rb proteins, respectively. None of the clinicopathological factors correlated with p16^{INK4}, cdk4, or Rb expression separately. A low p16^{INK4}/cdk4 ratio was significantly associated with a high disease stage ($p = 0.04$), and the ratio tended to be lower in mucinous than nonmucinous tumors. BACs

with a low p16^{INK4}/cdk4 ratio showed significantly higher Rb expression levels ($p = 0.02$). Univariable survival analyses showed a significantly lower 5-year survival probability in patients with a high stage ($p = 0.002$) or low p16^{INK4}/cdk4 ratio ($p = 0.01$). **Conclusions:** The results suggest a role of the cdk4/p16^{INK4} pathway in the prognosis of BACs. Further studies are warranted to clarify whether a low p16^{INK4}/cdk4 ratio may identify tumors that are destined to behave unfavorably.

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Introduction

Many studies have shown that the growth rate differs markedly among tumors [1–3], and that it closely correlates with prognosis [1–4]. Hence, it is unclear which factors contribute most to the heterogeneity in the growth potential among tumors.

Cyclins and cyclin-dependent kinases (cdks) are important regulators of the cell cycle progression during the first gap (G1) phase of the cell cycle. Some cyclins, such as cyclin D and E, have been suggested to be oncogenes or tumor promoter genes, and others such as inhibitors of

cdks, including the p16^{INK4} protein, to be tumor suppressor genes. The p16^{INK4} protein has been identified as a potent inhibitor of cdk4 by blocking cdk4-mediated phosphorylation of the tumor suppressor retinoblastoma (Rb) protein and also Rb-related proteins [5]. Phosphorylation of the Rb protein suspends Rb-mediated growth inhibition, thus allowing the cell to enter into the S phase of the cell cycle [5, 6]. The gene encoding p16^{INK4} has been identified as CDKN2/MTS1 gene, cloned from the short arm of the human chromosome 9 [6], and its mutation has been found in many tumors, including melanomas, leukemias, gliomas and non-small cell lung cancers (NSCLC) [6–8]. It has been further shown that a loss of p16^{INK4} is essential for maintenance of the transformed phenotype [9]. These properties of p16^{INK4} protein suggest that it is a tumor suppressor gene product. Consequently, cells unable to produce p16^{INK4} protein may be liable to neoplastic transformation. Bronchioloalveolar carcinomas (BACs) comprise a distinct category of lung adenocarcinomas with an incidence rate as high as 24% of all lung cancers [10]. Although BAC is a low-grade malignancy and generally has a favorable prognosis, its biological behavior is variable and the postoperative outcome is unpredictable. The presence of multifocal tumor and aerogenous spread are usually indicative of poor prognosis.

Several studies have assessed the role of p16^{INK4} protein in NSCLC using mixed populations of various types and stages of the tumors and found a frequent loss of p16^{INK4} in both the early and late stages of the disease [11, 12]. A strong association between aberrant p16^{INK4} expression and worse survival was also observed [11]. In a preliminary study of cdk4 and p16^{INK4} expression in BACs (unpubl. observation), we noted that BACs frequently expressed p16^{INK4} and that the ratio of p16^{INK4}/cdk4 rather than their individual values appeared to be potentially more informative. These data in addition to the lack of information on the status of p16^{INK4}, cdk4, and Rb proteins in BACs per se prompted us to assess the prognostic value of these cell cycle regulators in BACs. Furthermore, we had a collection of 38 BACs available which provided a considerable number of cases with this histological subtype for a comprehensive study. The nature of BACs as highly differentiated and usually low-stage tumors makes them a suitable material for studying the functional balance between cdk4 and its inhibitor p16^{INK4} protein, and their potential prognostic role as well. Moreover, information on the prognosis of these tumors may be clinically more applicable, because survival in advanced lung cancer patients is so uniformly poor that aggressive therapy is the rule in every case.

Using immunohistochemical methods on serial tissue sections from BACs, we evaluated the expression and interrelationship of p16^{INK4}, cdk4, and Rb proteins and correlated the findings with known clinicopathological factors of the disease to clarify their potential role in this disease.

Patients and Methods

Tissues

Thirty-eight consecutive tumors obtained by curative surgical resections for BACs were studied after informed consent had been obtained. Of the 38 patients, 23 (61%) were female and 15 (39%) were male, 31 (82%) were nonsmokers and 7 (18%) were smokers. The median age of the patients was 65 years (range: 48–78 years). All tumors in this cohort were solitary, located at the lung periphery, and ranged in size from 10 to 57 mm in the largest diameter with a mean of 25 mm (SD ± 9.53). Histologically, the tumors had been designated as BACs and classified according to the standard criteria described previously [13]. The presence of aerogenous spread was not observed in this cohort of patients. Twenty-nine (76%) of the BACs were nonmucinous, 7 (19%) were mucinous, and 2 (5%) were sclerosing by the histological type, and 34 had stage I (T1N0M0), 1 had stage II (T1N1M0), and 3 had stage III (T1–3N2M0) disease. No patient had received postoperative adjuvant therapy. The median follow-up time of the patients was 63 months (range 31–138). Overall, 32 patients were alive with no evidence of disease and 6 patients were dead at the time of analysis.

Antibodies, Immunohistochemistry, and Interpretation

Rabbit polyclonal antibody raised against a peptide corresponding to amino acid 282–303 mapping at the carboxyl terminus of cdk4 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA), mouse monoclonal antibody PMG3–245 raised against a Trp-E-Rb fusion protein recognizing an epitope between amino acids 332–344 of the human retinoblastoma protein, pp110–114 Rb (PharMingen, San Diego, Calif., USA), and rabbit polyclonal anti-p16^{INK4} antibody raised against a full-length recombinant bacterially produced GST-p16^{INK4} fusion protein (PharMingen) were used. The specificity of the antibodies had been confirmed by Western blot and immunohistochemical analyses in previous studies [14–16], and in the present study as well (data not shown). Serial 4- μ m-thick sections from the lesions mounted on silane-coated glass slides were stained using a streptavidin-biotin-peroxidase method [14]. For cdk4 and Rb, but not p16^{INK4}, an antigen retrieval step was applied by placing the sections in 0.1 M Tris-HCl buffer (pH 10.0) and heating in a microwave oven up to boiling, then cooling at room temperature for 30 min. Negative controls included omission of the primary antibody, its preabsorption with the corresponding peptide, or its substitution with nonimmune rabbit or mouse serum. Internal controls were positive nuclear staining of stromal cells and lymphocytes present in each section, as well as sections from colon and ovarian carcinomas known to be positive for p16^{INK4}, cdk4, or Rb proteins. In addition, the reliability of immunostaining was confirmed by comparable results for the proteins on immunoblotting and immunostaining in the known negative and positive tissues. The interpretation of staining was done based on the nuclear staining for p16^{INK4}, cdk4, and Rb proteins since the

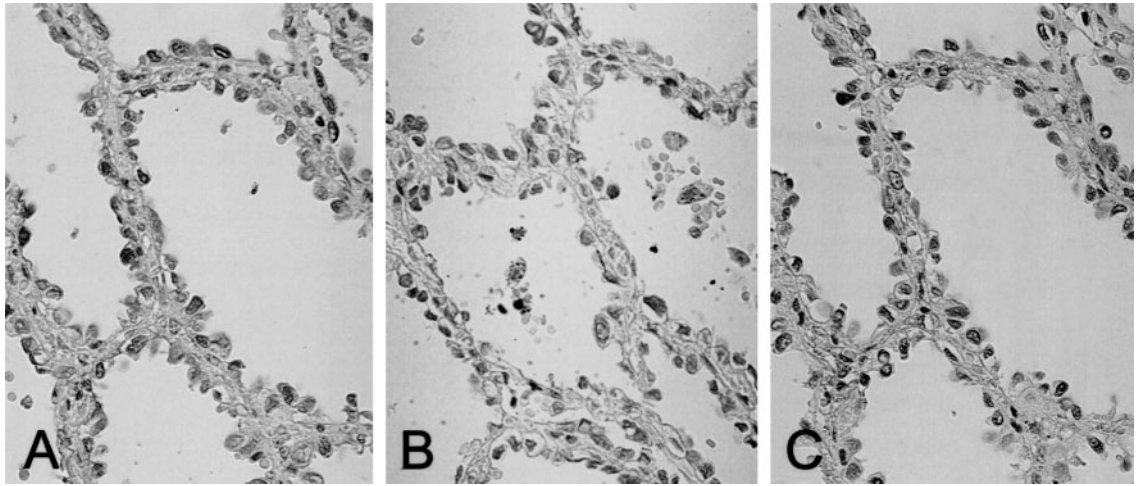


Fig. 1. Representative areas from serial tissue sections of a BAC showing abundant staining for p16^{INK4} (A) and cdk4 (C), and scant staining for Rb (B) proteins in tumor cell nuclei. Magnification ×450.

nature of cytoplasmic staining is controversial [14]. In fact, a very low number of the tumor cells in our series showed weak cytoplasmic staining. All interpretations were performed blindly without the knowledge of the clinical status of the patients. Immunostaining was assessed by the percentage of the stained tumor cell nuclei derived from the enumeration of more than 500 tumor cells in comparable areas in serial sections at 400× magnification and classified as score 0: <5%, score 1: 5–25%, score 2: 26–50%, score 3: 51–75%, and score 4: >75% of positive nuclei. This method of assessment has been widely accepted and used in previous studies [17]. Subsequently the ratio of p16^{INK4} score over the cdk4 score was calculated for each case (range: 0–4).

Statistics

Fisher's exact test was used to determine the statistical significance of differences of the clinicopathological factors according to the expression of p16^{INK4}, cdk4, and Rb proteins as well as the p16^{INK4}/cdk4 ratio. Univariable survival analysis was performed using the Kaplan-Meier method and compared by the log-rank test. Statistical analyses were performed by the SPSS statistical software system (SPSS, Chicago, Ill., USA). The degree of significance was set at $p < 0.05$.

Results

The nuclei of normal alveolar epithelial cells associated with the tumor in the tissue sections occasionally showed weak focal staining for p16^{INK4}, cdk4, or Rb proteins, whereas their cytoplasm were uniformly negative. In all instances, some stromal cells or lymphocytes in addition to tumor cells showed positive nuclear staining, indicating a good preservation of the antigenicity (fig. 1). Positive controls showed various degrees of staining for

p16^{INK4}, cdk4 and Rb proteins, and negative controls demonstrated no immunoreactivity. Of the 38 BACs, 34 (89%), 38 (100%), and 31 (82%) expressed p16^{INK4}, cdk4, and Rb proteins, respectively. The absence of p16^{INK4} expression (<5% positive nuclei) was observed in only 4 cases (11%). The median semiquantitative values for p16^{INK4}, cdk4, Rb, and p16^{INK4}/cdk4 ratio were set to divide the cases into two groups of low and high expressions for statistical comparison. None of the clinicopathological factors correlated with individual expression of p16^{INK4}, cdk4, or Rb protein. A low p16^{INK4}/cdk4 ratio was significantly associated with a high disease stage ($p = 0.04$) and the ratio tended to be lower in mucinous (median: 0.3; range: 0–1) versus nonmucinous (median: 1.0; range 0–1) tumors at a marginal significance ($p = 0.06$) (fig. 2). As an inverse correlation between the expression of p16^{INK4} and Rb proteins had been reported previously [10, 17], we also assessed the status of Rb protein in the two groups. BACs with a high p16^{INK4}/cdk4 ratio frequently showed no or traces of staining for Rb protein (14 of 22; 64%) as compared with BACs with a low p16^{INK4}/cdk4 ratio (4 of 16; 25%; $p = 0.02$). Univariable survival analysis in relation to the clinicopathological factors is listed in table 1. The overall 5-year survival probability of the patients in this cohort was 84.7%. The 5-year survival probability was significantly lower in patients with high-stage (II–III) disease (survivors, $n = 1$) than low-stage (I) disease (survivors, $n = 31$; $p = 0.002$), and in patients with a low p16^{INK4}/cdk4 ratio (survivors, $n = 11$) than a high ratio (survivors, $n = 21$; $p = 0.01$; fig. 3).

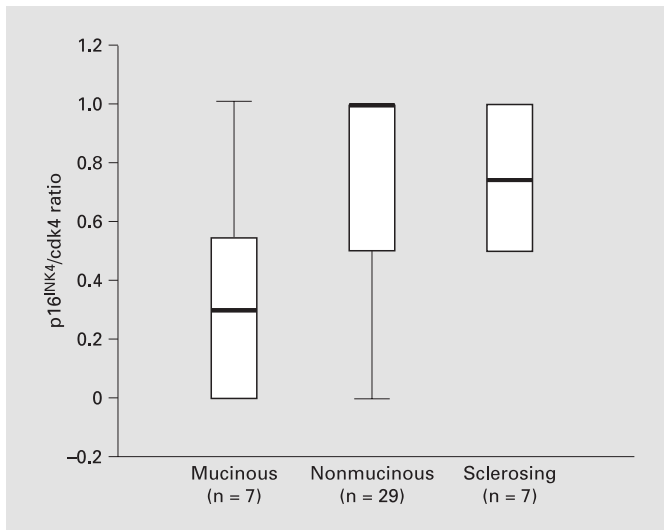


Fig. 2. Box plot of the p16^{INK4}/cdk4 expression ratios according to the histological subtypes. Median (thick line through the boxes), quartile, and extreme values within each group are shown. There is a tendency of a lower ratio in the mucinous type at a marginal significance of $p = 0.06$.

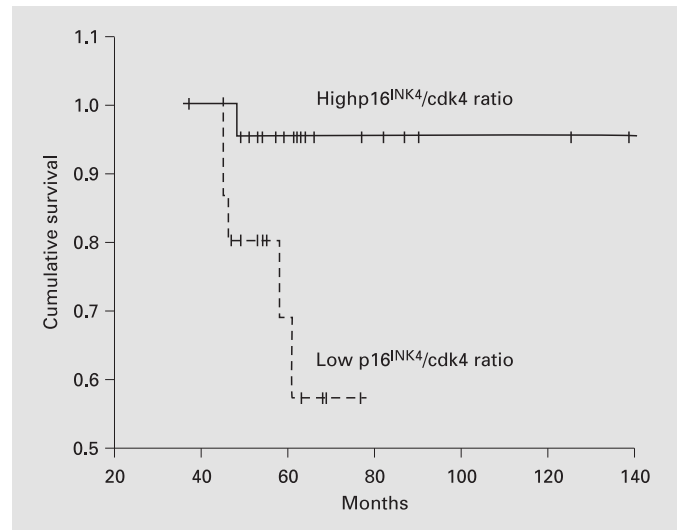


Fig. 3. Cumulative survival curves of the patients with high and low p16^{INK4}/cdk4 ratios with the Kaplan-Meier method as compared by the log-rank test shows a significant difference ($p = 0.01$) between the two groups.

Discussion

The present study demonstrated a role of the cdk4/p16^{INK4} pathway in the prognosis of BACs, by implying that a low p16^{INK4}/cdk4 ratio may predict BACs that are destined to behave unfavorably. We also showed that only 11% of BACs lost p16^{INK4} expression as compared with more than 50% in non-BAC NSCLC [9, 11, 15]. A greater abundance of p16^{INK4} expression in BACs is in contrast to its frequent loss observed in other types of tumors with higher malignant activity [8, 15], and thus points to a potential role of p16^{INK4} expression in defining more favorable biological behavior. Moreover, we verified the status of Rb in BACs with different p16^{INK4}/cdk4 ratios, and found that Rb-positive BACs frequently showed a low p16^{INK4}/cdk4 ratio, while Rb-negative BACs demonstrated a high p16^{INK4}/cdk4 ratio. A high synthesis of p16^{INK4} protein can inhibit the cdk4-mediated phosphorylation of the tumor suppressor Rb protein, thus allowing Rb-mediated growth suppression [5].

Although several studies on cyclins, cdks, and inhibitors of cdks have been reported in NSCLC [9, 11, 12, 16, 18] there is no study confined to BACs, except a recent report examining p21 and cyclin D1 expressions in 19 patients with BACs [19]. In this report, nuclear expression of cyclin D1 and p21 was found in 6 (32%) and 9 (47%) patients, respectively. However, no significant association

Table 1. Univariable survival analysis of the BAC patients in relation to the clinicopathological variables

Variable	Patients	Events	5-year survival probability, %	p value
All patients	38	6	84.7	-
Age, years				0.73
<60	12	2	80.0	
≥60	26	4	85.1	
Tumor size, mm				0.11
≤20	17	1	92.9	
>20	21	5	74.7	
Stage				0.002
I	34	3	90.7	
II-III	4	3	50.0	
Histological type				0.35
Nonmucinous	29	3	85.7	
Mucinous	7	2	83.3	
Sclerosing	2	1	50.0	
p16 ^{INK4}				0.10
Low (≤1)	21	5	74.3	
High (>1)	17	1	93.3	
cdk4				0.69
Low (<2.5)	19	1	85.7	
High (≥2.5)	19	5	82.5	
Rb				0.80
Low (<2)	20	3	83.6	
High (≥2)	18	3	84.4	
p16 ^{INK4} /cdk4 ratio				0.01
Low (<0.6)	16	5	68.5	
High (≥0.6)	22	1	95.2	

was found with smoking habit, tumor size, disease stage, and mucinous versus nonmucinous subtype of the tumors. It has been shown that cyclin D1 stimulates cdk4-mediated phosphorylation of the Rb protein which inactivates its growth-inhibitory function [20]. It has also been shown that NSCLCs, including BACs, are predominantly Rb positive, and might require lesser amounts of functional p16^{INK4} protein in order to activate levels of cdk4 activity sufficient for Rb inactivation [9]. Therefore, Rb-positive NSCLC might overexpress cyclin D1 in order to inactivate Rb, and has shown little or no p16^{INK4} protein, while Rb-negative NSCLC might not require excessive cyclin D1, and has demonstrated abundant p16^{INK4} protein [9]. Furthermore, discrepancies exist with regard to the expression of p16^{INK4} and cdk4 proteins in relation to the conventional clinicopathological prognostic factors of malignant tumors. While no significant correlation has so far been reported between the expression of cdk4 and clinicopathological factors, some authors have found that increased p16^{INK4} expression is associated with poor prognosis, for example in ovarian carcinoma [21] and prostate cancer [22]. In contrast, others have reported that decreased or loss of p16^{INK4} expression is an indicator of poor prognosis, for example in melanomas [23], pancreatic carcinoma [24], and NSCLC [25]. Although the discrepancies in the results may be explained by the differences in the methodology, the positive or negative Rb status of the tumors might have played a major role. In the light of these contradictory findings, and since the p16^{INK4} and cdk4 proteins are reciprocally inversely functioning, it appeared more meaningful to evaluate the ratio of p16^{INK4}/cdk4 expression in the same tissue rather than their individual expression levels.

In our study, the expression of p16^{INK4}, cdk4, or Rb separately did not correlate with any of the clinicopathological factors of BACs such as patients' age, tumor size, disease stage, and histological subtype. However, a low p16^{INK4}/cdk4 ratio was significantly associated with a high disease stage and a shorter survival time. These results suggest that a low p16^{INK4}/cdk4 ratio may reflect a more aggressive phenotype of tumor cells, and thus might play an imperative role in predicting the biological behavior of BACs. The present observations are supported by previous studies showing a strong association between the loss of p16^{INK4} expression and worse survival [11] and increased incidence of mutations in the p16^{INK4} gene in metastatic lesions [12]. The finding that BACs have frequently shown p16^{INK4} expression is in accord with a lower incidence of loss of p16^{INK4} expression previously observed in adenocarcinomas compared to other histolog-

ical subtypes of NSCLC [11]. In our study, the univariable statistical significance of the relationship between p16^{INK4}/cdk4 ratio and survival is based on a relatively small number of events occurring in our series (n = 6). Accordingly, multivariable statistics to assess independent significance was inapplicable. Therefore, we emphasize the need for further independent verification of this interesting relationship. It is also worthwhile to assess the value of the p16^{INK4}/cdk4 ratio as a marker for predicting the response to cytotoxic therapy in cases who need this mode of therapy.

Among the three histological subtypes of BACs, mucinous tumors are the rarest form and known to carry a significantly worse prognosis compared to the nonmucinous form [13]. Our finding that mucinous BACs had a higher tendency toward a low p16^{INK4}/cdk4 expression ratio may lend additional support to this concept.

In conclusion, our study has shown that the biological behavior of BACs might be reflected in their expressional ratio of p16^{INK4}/cdk4 proteins. BAC patients with prolonged survival time had a significantly higher p16^{INK4}/cdk4 ratio than those with shorter survival times (p < 0.01), suggesting a potential role of the p16^{INK4} expression in defining a favorable prognosis. Recent studies have also shown a favorable independent prognostic role of p16^{INK4} expression in NSCLC patients [18, 26].

Taken together, our results suggest the involvement of a cdk4/p16^{INK4} pathway in the prognosis of BACs. Further studies are required to assess whether a low p16^{INK4}/cdk4 ratio may predict BACs that are destined to behave unfavorably. If so, strategies designed to restore p16^{INK4} expression or function could represent a novel therapeutic approach to this disease.

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