

ORIGINAL ARTICLE

Absence of prognostic significance of p21^{WAF1/CIP1} protein expression in non-small cell lung cancer

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Abstract

Prognostic value of p21^{WAF1/CIP1} expression in non-small-cell lung cancer patients (NSCLC) remains unclear. In this study the authors investigated the clinical significance of p21^{WAF1/CIP1} expression in a group of 117 NSCLC patients, who underwent curative pulmonary resection. Expression of p21^{WAF1/CIP1} protein was assessed immunohistochemically and samples showing >5% of positive tumor cells were considered positive. Seventy-six samples (65%) showed positive nuclear p21^{WAF1/CIP1} protein expression. There was no relationship between the expression of p21^{WAF1/CIP1} protein and major clinico-pathological factors, and neither there was an impact of p21^{WAF1/CIP1} protein expression on disease-free and overall survival. p21^{WAF1/CIP1} protein occurrence was not correlated with previously determined p53 protein expression and there was also no relationship between all possible p21^{WAF1/CIP1}/p53 phenotypes and survival. In uni- and multi-variate analysis only stage of disease was independent prognostic factors. These results suggest the lack of prognostic relevance of p21^{WAF1/CIP1} expression (analyzed separately or jointly with p53 protein) in surgically treated NSCLC patients.

Introduction

The human *WAF1/CIP1* gene (6p21.2) encodes a 21 kDa protein, whose expression is directly induced by wild-type p53 protein [1]. p21^{WAF1/CIP1} binds to a variety of cyclin-dependent kinases inhibiting their activity [2], regulates DNA repair and by inhibiting the proliferating-cell nuclear antigen directly blocks DNA replication [3]. p21^{WAF1/CIP1} is additionally linked to terminal differentiation [4], senescence [5], and inhibition of apoptosis [6,7]. p53-dependent induction of p21^{WAF1/CIP1} causes cell cycle arrest after DNA damage [1]. In tumor cells, in the case of loss of the p53 protein or presence of an altered form of p53, p21^{WAF1/CIP1} level is dramatically reduced or totally absent [8]. Moreover, p21^{WAF1/CIP1} can be regulated independently of p53 [9] in several situations includ-

ing cellular differentiation and normal tissue development [4].

The clinical significance of p21^{WAF1/CIP1} expression in non-small cell lung cancer (NSCLC) remains unclear. Among many clinical studies on p21^{WAF1/CIP1} in NSCLC [10–22] only a few have been focused on correlation between p21^{WAF1/CIP1} expression and survival [18–22]. Therefore the aim of this study was to determine whether p21^{WAF1/CIP1} protein expression gives any additional prognostic information in NSCLC patients managed with curative resection. Since most of the tumors used in this study were previously assayed for their p53 protein status, we additionally performed analysis of both markers' co-expression and investigated the impact of their various combinations on treatment outcome [23].

Material and methods

The study group included 117 NSCLC patients who underwent curative pulmonary resection at the Department of Thoracic Surgery, Medical University of Gdańsk, Poland, between 1994 and 2001 (Table I). None of the patients had undergone preoperative treatment; three (2%) and eight (7%) of them received adjuvant chemotherapy and radiotherapy, respectively.

Tissue specimens were taken intraoperatively, formalin fixed, and paraffin embedded. Hematoxylin–eosin stained tissue sections were classified and differentiated according to the revised classification of the WHO [24]. Determination of the stage of disease (pTNM) was based on the current UICC criteria [25], after pathological examination of primary tumor and regional lymph nodes.

p21^{WAF1/CIP1} protein expression was assessed using monoclonal antibody (clone SX 118, DakoCytomation Sp.z o.o., Gdania, Poland) and the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method (Figure 1).

Tumor samples (3–5 µm) were cut and air-dried overnight before staining at 50°C. Sections were dewaxed in xylene and rehydrated through graded

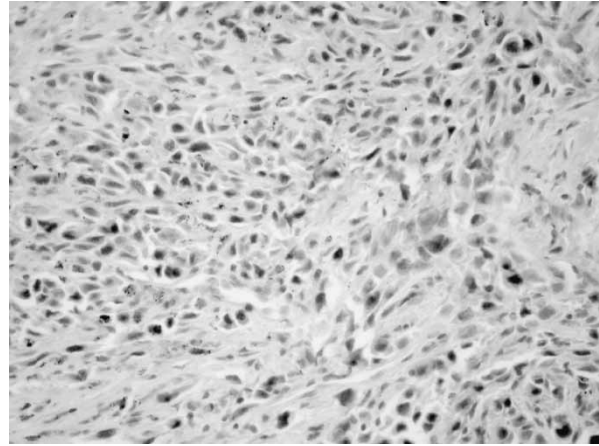


Figure 1. Positive staining for p21^{WAF1/CIP1} protein (clone SX 118, magnification 400 x).

alcohols (99%, 96%, 75%). The pretreatment procedure consisted of incubation in citrate-buffer pH 6.0 for 30 min at 95°C. Then, the slides were cooled down to room temperature for 20 min and dipped in TBS for 5 min. Next power-block diluted 1:10 was dropped on tissue scrap and left for 10 min. The p21^{WAF1/CIP1}, diluted 1:50, was applied overnight. The APAAP complex was subsequently applied for 30 min. The reaction was developed with alkaline

Table I. Correlation between p21^{WAF1/CIP1} and p53 expression and clinical variables (χ^2 test).

Variables	n = 117 (%)	p21 ^{WAF1/CIP1} expression (%)	p
Age			
≤60 years	49 (42)	31 (63)	0.747
>60 years	68 (58)	45 (66)	
Sex			
Male	91 (78)	60 (66)	0.682
Female	26 (22)	16 (62)	
Stage			
I	48 (41)	34 (71)	0.136
II	16 (14)	9 (56)	
IIIA	45 (38)	30 (67)	
IIIB+IV	8 (7)	3 (38)	
Histology			
Squamous	79 (67)	54 (68)	0.789
Adenous	22 (19)	9 (41)	
Large cell	8 (7)	7 (88)	
Adeno-squamous	8 (7)	6 (75)	
Grade			
G1	17 (14)	13 (76)	0.578
G2	66 (57)	37 (57)	
G3	34 (29)	26 (76)	
p53 status			
Negative	61 (52)	39 (64)	0.811
Positive	56 (48)	37 (66)	

phosphatase substrate containing naphthol AS-BI phosphate Na-salt and N-N dimethylformamid. Slides were then counterstained for 1–2 min. with haematoxylin.

The presence of p53 was assessed as previously described [23] with the use of Ab-2 mouse monoclonal antibodies—clone PAb1801 (Oncogene Science, Merck Sp. z o.o., Warsaw, Poland) and APAAP method.

Two independent observers (C.B and K.H.W.) assessed the immunostaining. Samples showing >5% of positive cells were regarded as positive for both p21^{WAF1/CIP1} and p53 protein.

Statistics

Chi² and Fisher’s tests were used to assess the relation between p21^{WAF1/CIP1} and clinical characteristics. Survival curves were calculated according to the Kaplan–Meier method and compared with the use of the log-rank test. Multivariate analysis was based on the Cox regression model with p values lower than 0.05 considered as statistically significant.

Results

Nuclear expression of p21^{WAF1/CIP1} protein was found in 76 (65%) of tumor samples. Mean percentage of p21^{WAF1/CIP1} expression in all positive tumors was 24%. In χ^2 analysis there was no correlation between p21^{WAF1/CIP1} expression and clinical variables (Table I). There was also no correlation between p21^{WAF1/CIP1} and p53 protein expression even if various cut-off values were taken (0%, 1% and 10%). Only five patients (4%) in this series were non-smokers, therefore analysis for smoking habit was not performed.

Survival analysis was performed in 106 patients; 11 patients with IIIB and IV stage of disease were excluded from this analysis due to incompleteness of surgical resection. At the time of this analysis (January 2004), with a median follow-up of 62 months (range from 37 to 113), 44 patients were alive (40%). Median overall survival (OS) for the entire group was 34 months and one- and five-year

OS probability was 70% and 42%, respectively. Median disease-free survival (DFS) for the entire group was 28 months, and one- and five-year DFS probabilities were 59% and 37%, respectively.

Median OS for patients with and without p21^{WAF1/CIP1} expression was for both groups 34 months, and 5-year survival probability was 43% and 39%, respectively (log-rank, p = 0.92). Median DFS for patients with and without p21^{WAF1/CIP1} expression was 28 and 29 months, respectively and 5-year DFS probability was 40% and 30%, respectively (log-rank, p = 0.80). There was no significant difference in OS and DFS between particular groups of patients with all possible p21^{WAF1/CIP1} /p53 phenotypes (Table II, Figure 2). In multivariate analysis only stage of disease was an independent prognostic factor for DFS and OS (Table III). There was also no influence of p21^{WAF1/CIP1} protein expression on OS and DFS (considered separately or jointly with p53) even if various cut-off values were taken for both proteins (0%, 1% and 10%).

Discussion

Although many studies on p21^{WAF1/CIP1} status have been conducted in lung cancer [10–22], only a few address prognostic relevance of this marker in NSCLC and their results are not consistent [18–22]. The most important information deriving from this study is that p21^{WAF1/CIP1} expression probably gives no prognostic information in surgically treated NSCLC patients. This finding remains consistent with the report of Komiya et al. [18] and Bennett et al. [20], both sized similar to our series (by Komiya et al. n = 137 and by Bennett et al. n = 106). However, Komiya et al. stated also that patients with squamous cell lung cancer and p21^{WAF1/CIP1} expression survived longer [18]. In other studies [19,21,22] p21^{WAF1/CIP1} expression was correlated with better prognosis in all types of NSCLC. Additionally, in one of them patients with p21^{WAF1/CIP1}+/p53 – phenotype lived longer in correlation to other patients (p = 0.001) [21] but other studies, including ours, did not find such a correlation [18–20].

Table II. The impact of p21^{WAF1/CIP1} expression and p21^{WAF1/CIP1}/p53 phenotypes on DFS and OS.

Phenotype	No.	p	Disease-free survival		Overall survival	
			p	Hazard ratio (95% CI)	p	Hazard ratio (95% CI)
p21 ^{WAF1/CIP1} –/p53 – vs. others	20	0.44		1.25 (0.65–1.86)	0.56	1.2 (0.57–1.83)
p21 ^{WAF1/CIP1} –/p53+ vs. others	17	0.33		0.72 (0.08–1.37)	0.74	0.88 (0.18–1.59)
p21 ^{WAF1/CIP1} +/p53 – vs. others	37	0.40		1.24 (0.73–1.75)	0.29	1.33 (0.79–1.87)
p21 ^{WAF1/CIP1} +/p53+ vs. others	35	0.43		0.81 (0.32–1.31)	0.18	0.7 (0.20–1.21)

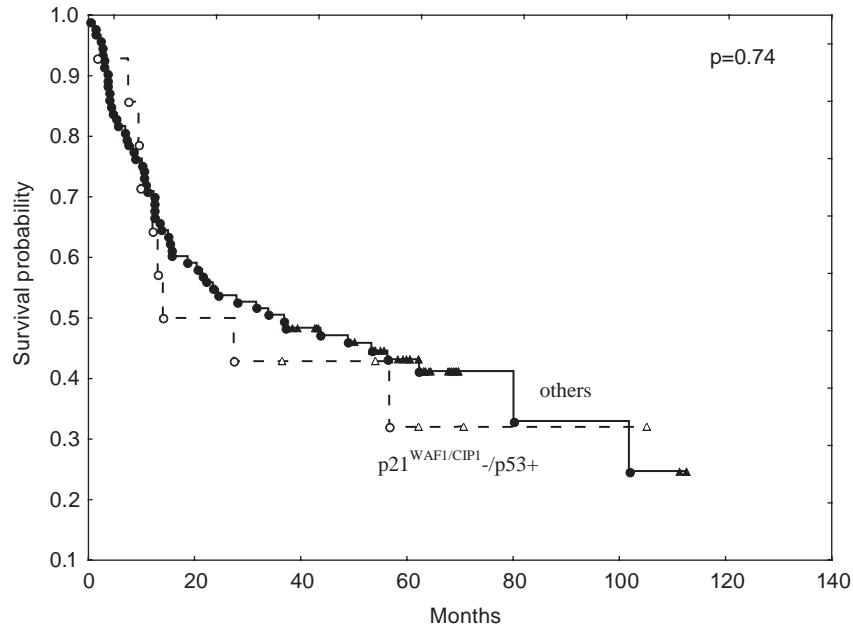


Figure 2. Overall survival of patients with $p21^{WAF1/CIP1} -/p53+$ protein expression vs. others.

In our series $p21^{WAF1/CIP1}$ protein expression was found in 65% of tumors, well in the range found in other series (27–80%) [10–22]. The range of $p21^{WAF1/CIP1}$ expression in particular studies is relatively large and this may be due to several factors, including the type of primary antibody or different cut-off values. Some authors considered as positive samples showing any $p21^{WAF1/CIP1}$ expression [20], others used 1% [16] or 10% cut-off value [13]. In our study, similarly to Akita et al. [17], Komiya et al. [18], Caputi et al. [19], and Shoji et al. [21], a 5% cut-off value of positivity was used. We decided to present this cut-off value to make our results more comparable; however, statistical analysis for different cut-off values (0%, 1% and 10%) was also performed.

In one study $p21^{WAF1/CIP1}$ protein was detected more frequently in patients with stage I and II in comparison with stage IIIA ($p=0.048$) [18] and in adenocarcinoma compared with squamous cell car-

cinoma ($p < 0.01$) [14]. However, in other series (including ours) there was no significant correlation between the $p21^{WAF1/CIP1}$ expression and age, sex, tumor histology, and grade or stage of disease [13,16,19–21].

In this study we addressed possible correlation between $p21^{WAF1/CIP1}$ and p53 expression because many experimental studies demonstrated that $p21^{WAF1/CIP1}$ was a downstream target of wild-type p53 [1]. Similarly to other authors no statistical correlation between $p21^{WAF1/CIP1}$ and p53 status was indicated [10–16,18,20,21]. These finding could support the hypothesis that activation of $p21^{WAF1/CIP1}$ is also possible through a p53-independent pathway [9]. However, in the future determination of *WAF1/CIP1* and *P53* statuses should be performed.

In conclusion it seems that $p21^{WAF1/CIP1}$ protein expression alone or in combination with p53 status does not provide prognostic information in surgically

Table III. Multivariate Cox proportional hazard model of disease-free and overall survival.

Variable	Disease-free survival		Overall survival	
	p	HR (95% CI)	p	HR (95% CI)
Age	0.94	0.99 (0.95–1.03)	0.72	1.00 (0.97–1.04)
Sex	0.75	0.91 (0.33–1.49)	0.86	1.05 (0.47–1.64)
Stage of disease	0.003	1.41 (1.19–1.64)	0.001	1.48 (1.25–1.72)
Grade	0.16	1.31 (0.93–1.70)	0.07	1.46 (1.05–1.87)
Histology	0.48	1.07 (0.88–1.27)	0.44	1.08 (0.88–1.29)
$p21^{WAF1/CIP1}$ status	0.92	0.97 (0.49–1.46)	0.98	1.01 (0.50–1.51)
p53 status	0.63	1.13 (0.64–1.62)	0.63	1.13 (0.62–1.64)

HR=hazard ratio, 95% CI=95% confidence interval.

treated NSCLC patients but this hypothesis should be confirmed in large studies and after standardization of immunohistochemistry and scoring system.

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