ORIGINAL PAPER

Michael A. Levesque · Mario D'Costa
Eleftherios P. Diamandis

p21WAF1 protein expression determined by quantitative immunoassay in relation to non-small-cell lung cancer aggressiveness

Abstract *Purpose:* p21WAF1, a cyclin-dependent kinase inhibitor, is an important mediator of the cell-cycle arrest and tumor suppression induced by the protein p53. Although alterations of the *p53* gene and its overexpression are frequent in most malignancies, including non-small-cell lung cancer (NSCLC), and may be associated with poor patient prognosis, the clinical utility of p21WAF1 expression in NSCLC has not been established. *Methods:* We have used a commercial enzyme-linked immunosorbent assay (ELISA) kit for p21WAF1 to test soluble extracts of 54 NSCLC specimens with known clinicopathological properties. *Results:* There was no correlation between p21WAF1 and p53 concentrations, the latter being determined by a time-resolved immunofluorometric assay developed in-house. Furthermore, p21WAF1 levels were not associated with patient age, tumor/node/metastasis (TNM) stage, lymph node metastasis, histological grade or type, or smoking history, in Mann-Whitney analysis. χ^2-tests, based on cutoffs equal to the 25th, 50th, or 75th percentiles of the p21WAF1 distribution, similarly did not reveal any statistically significant associations between p21WAF1 and other clinicopathological variables. Because of the small number of patients and the median follow-up of only 18 months, a meaningful survival analysis could not be performed. *Conclusion:* In summary, this preliminary study suggests that ELISA-quantified p21WAF1 levels in NSCLC extracts are weaker than p53 in terms of prognostic value and do not contribute to the further subclassification of patients.

Key words Enzyme-linked immunosorbent assay · p53 protein · WAF1 protein · Lung cancer · Prognosis

Abbreviations *NSCLC* non-small-cell lung cancer · *RR* relative risk

M. A. Levesque · E. P. Diamandis (✉)
Department of Pathology and Laboratory Medicine,
Mount Sinai Hospital, 600 University Avenue,
Toronto, Ontario M5G 1X5, Canada
e-mail: ediamandis@mtsinai.on.ca
Tel.: +1-416-586-8443
Fax: +1-416-586-8628

M. A. Levesque · M. D'Costa · E. P. Diamandis
Department of Laboratory Medicine and Pathobiology,
University of Toronto, Toronto, Ontario, Canada

M. D'Costa
Department of Laboratory Medicine,
St. Joseph's Health Centre,
Toronto, Ontario, Canada

Introduction

Although the prognostic value of the p53 tumor suppressor protein has been extensively studied in non-small-cell lung carcinoma (NSCLC) (Kirsch and Kastan 1998), the clinical implications of the expression levels of other genes that mediate the ability of p53 to induce cell-cycle arrest and apoptosis remain unclear. Among the first genes shown to be transcriptionally up-regulated by p53 was *p21WAF1* (CIP1/SDI1) (E1-Deiry et al. 1993), encoding a protein that binds and inhibits a variety of cyclin/cyclin-dependent kinase complexes (Xiong et al. 1993). Despite observations that expression of p21WAF1, like p53, can cause growth suppression of a variety of cell types (E1-Deiry et al. 1993), *p21WAF1* mutations rarely occur in human cancers (Shimazu et al. 1996), suggesting that derangement of p21WAF1 function may not contribute to clinical disease. However, because p21WAF1 protein expression has been shown to be highly variable in several tumor types, including NSCLC (Takeshima et al. 1998), and to be subject to both p53-dependent and p53-independent transcriptional regulation in these tissues (Marchetti et al. 1996), it may offer prognostic information independent of that provided by p53 alterations. A small number of studies have demonstrated associations between

immunohistochemically detected p21WAF1 expression and the status of established NSCLC prognostic factors (Marchetti et al. 1996; Hayashi et al. 1997; Komiya et al. 1997; Caputi et al. 1998), and fewer studies have examined the impact of p21WAF1 levels on patient disease-free or overall survival (Komiya et al. 1997; Caputi et al. 1998). The purpose of this study was, therefore, to determine the relationships, if any, between p21WAF1 protein expression levels, quantified for the first time by an enzyme-linked immunosorbent assay (ELISA) method, and clinicopathological features of 54 patients with NSCLC.

Materials and methods

Lung cancer patients

A group of 54 patients undergoing operation for treatment of primary NSCLC at St. Joseph's Health Centre, Toronto, Ontario, Canada, between December 1993 and March 1995, were included in this study, which had received approval by the Ethics and Research Committee at this institution. These patients had been part of a larger cohort, studied previously for the prognostic impact of p53 overexpression (Levesque et al. 1998), from whom sufficient tumor tissue remained for extraction for p21WAF1 analysis. Prior to surgery, all patients had not been treated for lung cancer. Post-operatively, 16 patients received systemic chemotherapy, 5 were given radiotherapy, and 2 received both modalities. Patients were followed-up at the same institution for between 1 and 50 months (median = 18 months).

Tissue extraction and immunoassay analysis

Resected tumor tissues ($n = 51$) and biopsy specimens ($n = 3$) were snap-frozen and examined histologically, by which representative portions were selected for analysis. The percentage of tumor cells was estimated to be low (1%–33%), medium (34%–66%), or high (67%–100%) in 4, 21, and 29 cases respectively. As described previously (Levesque et al. 1998), crude cell lysates were prepared from approximately 200-mg samples of these specimens, which were assayed for total protein content, using a kit based on the bicinchoninic acid method (Pierce Chemical Co., Rockford, Ill.), and for both p53 and p21WAF1 proteins without knowledge of patient clinicopathological information. The immunofluorometric assay for p53, developed by the authors, employed p53-specific monoclonal (DO-1) and polyclonal (CM-1) antibodies and a sensitive time-resolved fluorescence detection system (Levesque et al. 1998). Previous comparison of this p53 immunoassay to immunohistochemical staining of matched lung tumor tissues revealed a high degree of concordance between the two methods (Levesque et al. 1997). p21WAF1 concentrations in the tumor extracts were determined by the WAF1 quantitative ELISA (Oncogene Research, Cambridge, Mass.), following the manufacturer's instructions. Features of this sandwich-type assay include a rabbit polyconal anti-p21WAF1 antibody immobilized to microtiter plates, a mouse biotinylated monoclonal antibody specific to p21WAF1 added after sample addition, and detection by streptavidin conjugated to horseradish peroxidase, which catalyzes the generation of a colored product, quantified by spectrophotometry (Labsystems, Helsinki, Finland). Dedicated software interpreted the resultant p21WAF1 concentrations from calibration curves constructed from the assay of lyophilized standards ranging in concentration from 0 to 20 U/ml. Extracts prepared from breast carcinoma cells (MCF-7 and T47-D), obtained from the American Type Culture Collection, cultured as described (Ozcelik et al. 1995) and for which the p21WAF1 status had already been determined (Ozcelik et al. 1995), were assayed in parallel as qualitative positive and negative controls respectively. To adjust for the variable extraction efficiencies, p53 and p21WAF1 concentrations were divided by the total protein contents of the extracts and were thereby expressed as ng/mg p53 and U/mg respectively.

Statistical analysis

All statistical procedures were performed by SAS for Windows version 6.12 software (SAS Institute, Cary, N.C.); they were nonparametric and based on two-sided tests of significance. Correlation between p53 and p21WAF1 was assessed by calculation of the Spearman correlation coefficient. Relationships between p21WAF1 concentrations and patient gender, age, stage, grade, histotype, node status, smoking history, and whether each of the clinical endpoints was reached during follow-up were examined by Mann-Whitney tests, as well as by χ^2-tests applied to contingency tables in which p21WAF1 status (positive or negative) was defined by 25th, 50th, or 75th percentile decision thresholds. Survival analysis was performed by univariate Cox proportional-hazard regression, in which all variables evaluated were collapsed into two-level variables from which the relative risks (RR) of relapse or death were estimated.

Results

Clinicopathological characteristics of the 54 NSCLC patients are given in Table 1. Protein-adjusted p21 WAF1 concentrations, which ranged from 0 to 5.1 U/mg and were distributed with a mean, standard deviation, and median of 1.44, 1.23, and 1.21 U/mg respectively (Fig. 1), were found by Spearman correlation analysis not to be correlated with concentrations of p53 ($r = 0.13$, $P = 0.34$; Fig. 2), which exhibited a wider range of values from 0.162 ng/mg to 142.57 ng/mg (mean = 14.76 ng/mg, standard deviation = 30.07 ng/mg, and median = 1.55 ng/mg). p21WAF1 concentrations also did not correlate with patient age ($r = 0.02$, $P = 0.88$) or the number of pack-years of cigarette smoking ($r = 0.07$, $P = 0.61$), nor were they significantly associated with these variables and others (including gender, disease stage, histological grade, histological type, lymph node metastases), or whether or not the patients relapsed or died during their respective follow-up periods, by Mann-Whitney tests (Table 1) or χ^2-tests applied to contingency tables in which p21WAF1 positivity was defined by 25th percentile (0.73 U/mg), median (1.21 U/mg), or 75th percentile (1.91 U/mg) cutoff points (data not shown). Univariate Cox proportional-hazard regression analyses did not reveal patients in p21WAF1-negative and p21WAF1-positive groups, categorized on the basis of each of the above cutoff points, to differ significantly in their RR for relapse of death (data not shown). In contrast are the abilities of other variables to demonstrate significant differences in risks for both survival outcomes: stage III–IV vs stage I–II (RR = 2.19, $P = 0.04$ for relapse and RR = 3.23, $P = 0.03$ for death), lymph node-positive versus node-negative (RR = 4.30, $P < 0.01$ for relapse and RR = 11.76, $P < 0.01$ for death), and p53 ≥ 1.55 ng/mg versus p53 < 1.55 ng/mg (RR = 3.28 $P < 0.01$ for relapse and RR = 2.63, $P = 0.04$ for death).

Table 1 Summary of clinicopathological variables and assessment of their relationships with p21WAF1 concentrations. *P* was determined by Mann-Whitney *U*-tests. Patients were grouped according to age on the basis of the median age, 66 years; their ages ranged from 42 to 83 years. The pathological stage was based on the tumor/node/metastasis (*TNM*) classification scheme, by which 29, 12, 9, and 3 patients were in stages I, II, III, and IV respectively; the stage was unknown for 1 patient. The histological grade was based on World Health Organization criteria, by which 4 tumors were G1, 32 were G2, and 15 were G3; the grade was unknown for 3 patients. Their history of tobacco use was known for 49 patients, of whom 43 had smoked an average of one pack of cigarettes per day for 46 years (range = 10–100 pack/years), while 6 patients were non-smokers. Recurrence implies documented evidence of recurrence or metastasis occurring from 1 to 30 months after surgery; the median disease-free survival time was 10 months. Deaths due to malignancy occurred from 1 to 40 months after surgery; the median overall survival time was 11 months

Variable	*n*	p21WAF1 concentration (U/mg) Median	Range	*P*
Sex				
Female	18	1.34	0.13–5.03	
Male	36	1.03	0–3.48	0.30
Age (years)				
<68	26	1.29	0.13–2.95	
≥68	28	0.99	0–5.03	0.89
TNM stage				
I–II	41	1.13	0–5.03	
III–IV	12	1.21	0.24–2.95	0.79
WHO grade				
1–2	36	1.03	0–5.03	
3	15	1.27	0–3.48	0.78
Histological type				
Adenocarcinoma	29	0.87	0–3.48	
Squamous cell carcinoma	25	1.27	0–5.03	0.37
Lymph node metastases				
No	31	1.30	0–5.03	
Yes	23	0.87	0–3.3	0.35
Smoking history				
No	6	0.83	0.34–1.4	
Yes	43	1.25	0–3.48	0.18
Recurrence				
No	30	1.15	0–5.03	
Yes	24	1.33	0.42–3.3	0.19
Death				
No	41	1.25	0–5.03	
Yes	13	1.06	0.42–2.95	0.85

The remaining variables – age, gender, histotype, and smoking history – were not statistically significant predictors in the survival analysis (data not shown).

Fig. 1 Distribution of p21WAF1 concentrations in the 54 lung tumor extracts
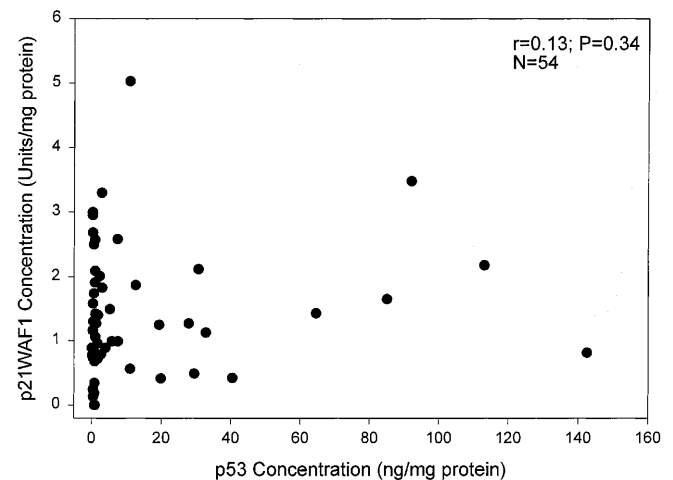
Fig. 2 Scatter plot of p53 and p21WAF1 concentrations

Discussion

The need for additional NSCLC prognostic indicators, particularly for patients with early-stage disease of whom approximately 50% have been shown to relapse within 5 years after undergoing curative resection, has led to the evaluation of molecular markers, such as p53 alteration, for their abilities to stratify NSCLC patients on the basis of risks of relapse or death. Because of the imperfect correlation between p53 mutation or subse-
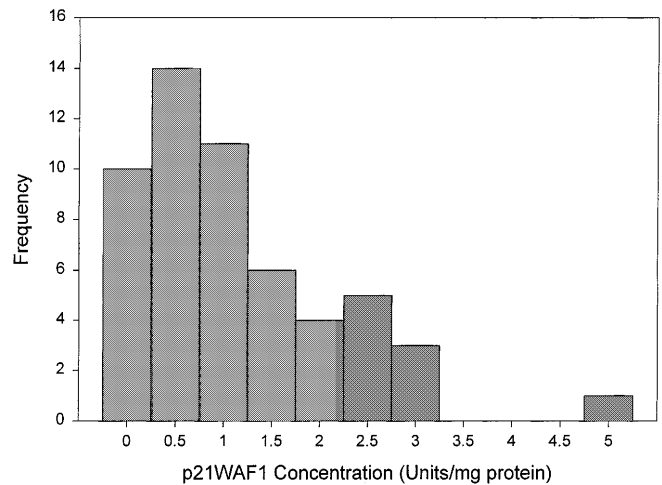
quent nuclear accumulation and the ability of p53 protein to function as a tumor suppressor (Kirsch and Kastan 1998), interest has also focused on the expression levels of p21WAF1, which is transcriptionally induced by wild-type p53 in response to cytotoxic stress and causes cell-cycle arrest (E1-Deiry et al. 1993; Xiong et al. 1993). Cellular expression of p21WAF1, reflecting to some extent the presence of functional p53, may therefore be associated with the same clinical and pathological features of NSCLC that are associated with wild-type p53.

On the basis of this assumption, and on the findings of others that p21WAF1 immunostaining was associated with favorable prognostic outcomes in a cohort of 60 NSCLC patients (Caputi et al. 1998) or only in a subset of another NSCLC series consisting of 48 patients with squamous cell carcinoma of the lung (Komiya et al. 1997), our inability to correlate p21WAF1 expression levels to the status of established prognostic factors or to

disease-free survival or overall survival was unexpected. This discordance could be due to methodological differences between studies and differences in the patients studied. In contrast to all previous studies of p21WAF1 protein expression in human cancers, which have used immunohistochemical techniques, we applied an ELISA method to extracts of lung tumor tissues. To our knowledge, this ELISA, although validated by the manufacturer and possibly providing more objective and quantitative results than conventional immunohistochemistry, has not been directly compared to immunostaining of matched lung tissue sections. Furthermore, important for interpreting the ELISA results obtained from tissue extracts is the tumor cellularity, for which significant differences between the specimens used in our study and those in earlier studies may be another factor. It is therefore possible that the relatively large proportion (46%) of lung tissue specimens with tumor cellularities below 67% may have yielded deceptively low p21WAF1 immunoassay results, given that other workers have shown little or no p21WAF1 expression in normal lung tissue (Marchetti et al. 1996; Hayashi et al. 1997; Takeshima et al. 1998) – findings, it must be noted, that we were unable to confirm because matched nonmalignant lung tissues were not available for the patients in our study. In other words, because the p21WAF1 concentrations were not adjusted for the variable tumor cellularities but rather only for the total protein contents of the extracts, a large number of specimens containing low percentages of p21WAF1-expressing tumor cells might have been assigned low p21WAF1 concentrations and consequently p21WAF1-negative status, potentially obscuring our ability to find statistically significant associations between p21WAF1 expression in NSCLC cells and the status of other clinicopathological parameters. Not investigated in this study was the possibility that immunoassay-quantified p21WAF1 expression might only be meaningful in those specimens with a large percentage of malignant cells. An additional difference between our study and those previously reported was that the follow-up for the patients in our study was shorter than in the previous two studies addressing the prognostic value of p21WAF1 (Komiya et al. 1997; Caputi et al. 1998). However, despite the variable tumor cellularities and the small number of patients and relatively short follow-up in our study, ELISA-determined p53 concentrations were found to be indicative of increased risks for relapse and death, unlike the p53 immunohistochemical staining results of Komiya et al. (1997) either in their whole cohort or in the subgroup of squamous cell carcinoma patients. These authors did not perform a statistical analysis of the relationship between p21WAF1 and p53 expression levels in NSCLC. In the present study, the correlation between these two variables was investigated and found to be weak, in accordance with other reports (Marchetti et al. 1996; Hayashi et al. 1997; Takeshima et al. 1998). In addition to p53, traditional NSCLC prognostic factors, such as pathological stage and lymph node status, also demonstrated strong associations with the disease-free and overall survival probabilities of our patient cohort.

In our study of 54 NSCLC patients, evaluation of three cutoff points for p21WAF1 positivity did not yield significantly different survival outcomes for p21WAF1-negative and -positive patients. Comparisons of risks for relapse or death between patients whose p21WAF1 concentrations fell in the extreme (first versus fourth) quartiles, although possibly revealing greater differences in prognosis, were not possible because of the small numbers of patients within these quartiles. Furthermore, we were unable to demonstrate statistically significant associations between p21WAF1 expression and NSCLC prognostic markers already in clinical use. Our findings suggest that the prognostic value of ELISA-quantified p21WAF1 expression in NSCLC is much weaker than that of p53 expression and does not contribute to further patient subclassification. Definitive confirmation of our findings, however, awaits further studies of larger numbers of NSCLC patients well-characterized with respect to the percentages of malignant cells in their specimens, post-operative treatment, long-term follow-up, and other clinicopathological factors related to prognosis.

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