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QUANTITATIVE ANALYSIS OF p53 PROTEIN IN NON-SMALL CELL LUNG CANCER AND ITS PROGNOSTIC VALUE

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Accumulation of mutant p53 protein occurs frequently in human malignancies, including 40–60% of non-small cell lung carcinomas. The implications of such *p53* over-expression, usually assessed by immunohistochemical techniques, for the prognosis of lung cancer patients remain undetermined. In this study, we used a time-resolved immunofluorometric assay to measure p53 protein concentrations in extracts prepared from 86 primary non-small cell lung tumours and examined the associations between p53 protein levels (corrected for total protein) and other clinico-pathologic variables, including post-surgical disease-free and overall survival. Contingency tables analysed by χ^2 tests revealed no significant relationships between p53 status, defined by a median cut-off point, and patient gender, age, disease stage, histologic grade and type, lymph node extension, smoking history and administration of adjuvant chemotherapy or radiation. However, multivariate Cox proportional hazard regression analysis demonstrated a dose-response relationship between p53 concentration, expressed as a 4-level, quartile-divided variable, and increased risk of relapse ($p = 0.010$) and death ($p = 0.016$). Patients whose tumours contained p53 concentrations exceeding the median value had over 3-fold higher risk of relapse ($p = 0.002$) and death ($p = 0.007$) than those whose tumours had lower p53 concentrations. We also provide evidence suggesting that the impact of p53 on survival is greater in patients with squamous cell carcinoma than in those with adenocarcinoma. Although the latter finding needs confirmation, our results suggest that application of an immunoassay of p53 protein on non-small cell lung tumour extracts may identify patients at increased risk of unfavourable outcome. ***Int. J. Cancer (Pred. Oncol.)*** **79:494–501, 1998.**
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Lung carcinoma is a leading cause of premature death in most countries and one of several malignancies for which the etiologic role of cigarette smoking has been clearly demonstrated. Unlike small cell lung cancer, which is less prevalent and typically disseminated at diagnosis but initially responsive to primary chemotherapy and radiotherapy, non-small cell lung cancer (NSCLC) accounts for approximately 85% of all cases and presents in one-third of these as localized disease treated primarily by surgery (Mountain, 1986). The prognosis of patients with advanced NSCLC is generally poor, and for this reason, adjuvant systemic therapy is usually instituted. Among patients with early-stage disease, however, 40–50% have been shown to relapse within 5 years after undergoing potentially curative resection (Mountain, 1986). Additional prognostic markers would be useful, particularly to identify those patients managed by surgery alone, who are at increased risk of early relapse or death and for whom adjuvant therapy may be beneficial.

Mutation of the *p53* gene has been shown to occur at very high frequencies in the majority of human cancers, including 40–60% of patients with NSCLC (Chiba *et al*., 1990; Kishimoto *et al*., 1992). These mutations, which are predominantly mis-sense and accompanied by nuclear accumulation of conformationally altered mutant p53 protein, have been detected in pre-neoplastic lesions of the lung (Sozzi *et al*., 1992; Bennett *et al*., 1993), in carcinomas *in situ* (Bennett *et al*., 1993) and in both primary tumours and metastases from individuals with extensive disease (Marchetti *et al*., 1993; Fontanini *et al*., 1994), suggesting that *p53* alteration may be an early event in NSCLC progression. Moreover, the high percentage of G-to-T transversion mutations of *p53* in lung tumours has been linked to exposure to carcinogens present in cigarette smoke (Suzuki *et al*., 1992). The cellular consequence of *p53* inactivation is thought to be the impairment of a pathway whereby cells harbouring damaged DNA are growth-arrested (Kastan *et al*., 1992) or deleted by programmed cell death (Lowe *et al*., 1993). A possible manifestation of this impairment in NSCLC may be resistance to chemotherapeutic agents, the efficacy of which is based on ability to induce *p53*-dependent apoptosis (Rusch *et al*., 1995). However, whether *p53* mutations predict shortened post-operative survival in these patients more accurately than the existing prognostic factors based on tumour morphologic characteristics remains undetermined, despite having been a subject of investigation for many years (McLaren *et al*., 1992; Quinlan *et al*., 1992; Mitsudomi *et al*., 1993; Lee *et al*., 1995; Passlick *et al*., 1995; Nishio *et al*., 1996).

Until quite recently, most studies of the prognostic implications of *p53* in NSCLC have focused on the detection of p53 protein accumulation in tumour tissue rather than on direct analysis of the *p53* gene for structural alterations. Although the simple immunohistochemical techniques commonly employed for this purpose have demonstrated good concordance with the results of DNA sequencing (Top *et al*., 1995), the different antibodies, specimen-processsing details and scoring criteria used for immunostaining in these studies have likely contributed to the discordant conclusions. Enzyme-linked immunosorbent assays (ELISAs) of p53 protein have also been described (Vojtěšek *et al*., 1992; Hassapoglidou *et al*., 1993; Levesque *et al*., 1995; Thomas *et al*., 1997) and may be advantageous in terms of sensitivity and specificity for the quantitative assessment of p53 protein in extracts of tumour tissues. With one exception (Pappot *et al*., 1996), these immunoassays have generally not been used to study the relationship between p53 protein accumulation status and the duration of survival in patients with NSCLC. Another such method, developed in our laboratory, has been used to demonstrate unfavourable prognosis associated with p53 protein over-expression in patients with breast carcinomas (Levesque *et al*., 1998) and has been shown to yield p53 protein concentrations in extracts of lung tumours, in agreement with the findings of immunostaining of the same tissues performed in parallel (Levesque *et al*., 1997). Since follow-up information, including that related to survival, was not initially available in the latter study, the prognostic value of ELISA-detected p53 protein in lung cancer could not be evaluated. The purpose of the present study was therefore to determine the relationships between p53

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protein concentration and risk of relapse and death in a subset of patients who were prospectively monitored for a median of 16 months and whose lung cancers were restricted to NSCLC histologic types.

PATIENTS AND METHODS

Lung cancer patients

In this study, we investigated a cohort of 86 patients operated at St. Joseph's Health Centre, Toronto, Canada, from July 1993 to March 1995 for treatment of primary NSCLC. The ethics and research committee at this institution had given approval. Excluded from the consecutive series were patients with multiple synchronous lung tumours or other primary malignancies metastatic to the lung, as well as patients whose lung tumours were of a non-invasive or small cell histologic type, were judged to be non-resectable or were of insufficient quantity for p53 protein analysis, and patients for whom follow-up information was unavailable. Surgery followed diagnosis of lung cancer by an average of 9 days and consisted of lobectomy (n = 46), pneumonectomy (n = 21), wedge resection (n = 12), lobectomy combined with wedge resection (n = 5) or biopsy obtained at bronchoscopy (n = 2). Ages of these patients, 58 of whom were male and 28 female, ranged from 42 to 87 years; the median age was 67 years. Among the 76 patients for whom history of tobacco use was known, the majority (n = 68) recounted smoking on average the equivalent of one pack of cigarettes per day for 45 years (range = 10–100 pack-years), while 8 patients remained non-smokers. Other clinico-pathologic features for which the majority of tumours had been characterised at the time of surgery included pathologic stage classification according to the tumour-node-metastasis (TNM) scheme (Beahrs *et al*., 1992) and the histologic grade and type based on World Health Organization (1982) criteria. Fifty-two patients (61%) were in stage I disease, 16 (19%) were in stage II, 13 (15%) were in stage IIIA, 1 (1%) was in stage IIIB and 3 (4%) had stage IV lung cancer. Of the 3 patients with stage IV disease, metastases to the bone and liver were identified in 2 cases and 1 case, respectively. Well-differentiated (G1) tumours comprised 7% (n = 6) of the specimens for which grade was known, while 62% (n = 51) were moderately differentiated (G2) and 30% (n = 25) were poorly differentiated (G3). Undifferentiated (G4) tumours were not present in our series. Tumour stage and grade could not be assessed for 1 and 4 patients, respectively. Most specimens were revealed to be either adenocarcinomas (n = 43, 50%) or squamous cell carcinomas (n = 37, 43%); the remainder consisted of large cell carcinomas (n = 5, 6%) and an adenosquamous carcinoma (n = 1, 1%). Regional lymph node metastases were identified in 32 of the patients.

For all patients, follow-up was performed by review of medical records at St. Joseph's Health Centre. Extended follow-up was also obtained by a questionnaire sent to other institutions and private practitioners throughout southern Ontario involved in the post-operative care. Informative responses were obtained for 56% of the questionnaires mailed. Forty patients (46%) underwent lung cancer relapse and 22 (26%) died of their disease during follow-up, which ranged from 1 to 44 months (median = 16 months). Disease-free survival time, defined as the interval between the dates of tumour resection and the first evidence of recurrent or metastatic disease, ranged from 1 to 30 months (median = 9 months). The corresponding period from surgery to death (the overall survival time) ranged from 1 to 40 months and had a median of 11 months. In the disease-free survival analysis, deaths without evidence of recurrence were considered censored observations, as were deaths due to causes other than lung cancer in the overall survival analysis (see below). Of the 61 patients remaining alive at last follow-up, 18 had relapsed and 43 were in remission. Prior to surgery, all patients had been untreated for lung cancer. Post-operatively, 13 patients received radiotherapy, 18 were administered systemic chemotherapy and 2 received both treatment modalities.

Tissue extraction and immunofluorometric assay of p53 protein

Immediately following surgical excision, lung tumour tissues were snap-frozen and examined histologically, by which representative portions were selected for subsequent analysis. The percentages of tumour cells in these specimens were judged to be low (1–33%), medium (34–66%) or high (67–100%) in 7, 34 and 45 cases, respectively. After storage at –80°C for no more than 2 months, approximately 200 mg of these tissues were pulverized to a fine powder on dry ice and combined with 1 ml of a cell lysis buffer [50 mM Tris, pH 8.0, containing 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40 surfactant, 10 mg/l phenylmethylsulfonylfluoride and 1 mg/l each of aprotinin and leupeptin], incubated for 30 min on ice and centrifuged for 30 min at 15,000 *g* at 4°C to collect the supernatants. The crude cell lysates were assayed directly for p53 protein using immunofluorometry and for total protein content using a kit based on the bicinchoninic acid method (Pierce, Rockford, IL).

A ''sandwich-type'' immunoassay of p53 protein, described in detail previously (Levesque *et al*., 1995), was used to measure p53 protein concentrations in the lung tumour extracts. This method employed 2 p53-specific immunoreagents, monoclonal DO-1 antibody immobilised to microtitre plates prior to sample addition and polyclonal CM-1 anti-serum (Novocastra, Newcastle-upon-Tyne, UK), added subsequently and detected by an alkaline phosphatase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). Enzyme activity was detected by monitoring the formation of fluorescent complexes between the dephosphorylated reaction product diflunisal and EDTA-chelated Tb^{3+}, using time-resolved fluorometry (Christopoulos and Diamandis, 1992) performed on a dedicated instrument (Cyberfluor-615 Immunoanalyzer; MDS Nordion, Kanata, Ontario, Canada). Assay calibrator solutions ranged in concentration from 0.15 to 75 ng/ml and were prepared by dilution of a lysate of Sf9 insect cells infected with a p53-expressing baculovirus (Levesque *et al*., 1995). The p53 protein concentration of this lysate had been determined by assay in parallel against purified, bacterially expressed p53 protein (Oncogene Research, Cambridge, MA). Concentrations of p53 protein in the lung tumour extracts exceeding the detection limit of approximately 0.04 ng/ml were divided by the total protein concentrations to adjust for differences in tissue mass and extraction efficiency. Samples and calibrators were run as duplicates and in parallel. Since the epitope recognised by DO-1 antibody is within the amino terminal domain shared by wild-type and the majority of mis-sense p53 mutants (Vojtěšek *et al*., 1992), the immunoassay is able to detect both mutant and wild-type p53 protein. Clinical specimens were assayed for p53 protein without knowledge of the corresponding clinico-pathologic or survival information.

Statistical analysis

All statistical procedures, performed by SAS for Windows version 6.12 software (SAS Institute, Cary, NC), were non-parametric and based on 2-sided tests of significance. Relationships between p53 protein concentrations and other continuous variables, such as age, number of pack-years smoked and the number of months until recurrence, death and last follow-up, were examined by calculation of Spearman correlation coefficients (r_s). These associations, as well as those between p53 and several categorical variables, were also investigated by χ^2 tests applied to contingency tables. In these tables, the distributions of p53-negative and p53-positive tumours (using a cut-off point equal to the median p53 concentration, 0.14 ng/mg) were compared between patients differing with respect to age (less than 66 years *vs.* greater than or equal to 66 years), pathologic stage (I–II *vs.* III–IV), histologic grade (G1–G2 *vs.* G3) and type (adenocarcinoma *vs.* squamous cell carcinoma *vs.* other), lymph node involvement (absent *vs.* present), smoking history (no *vs.* yes), post-operative treatment (not treated *vs.* treated with chemotherapy and/or radiotherapy) and whether each of the clinical end points was reached during follow-up (non-relapsed *vs.* relapsed and alive *vs.* dead of lung cancer). The prognostic roles of p53, alone and in conjunction with other

clinico-pathologic factors, in determining disease-free and overall survival were evaluated by hazards ratios [(HRs) relative risk (RR) of relapse or death] and 95% confidence intervals, which were calculated from fitted Cox proportional hazards regression models. In the multivariate analysis, the regression models were adjusted for age, stage, grade, node status and smoking history classified by the criteria given above. p53 protein concentration was tested in the models separately as a continuous variable; as a quartile-divided, 4-level ordinal variable; and as a dichotomous variable categorised by the median percentile cut-off point. Univariate and multivariate regression models were also used to evaluate the association between p53 protein concentrations exceeding the median value and post-surgical survival in the subgroups of patients diagnosed with adenocarcinoma, squamous cell carcinoma, early-stage (I–II) disease and late-stage (III–IV) lung cancer. p53-by-histotype and p53-by-stage interactions in the univariate Cox models were evaluated separately in order to determine if the effects of p53 on outcome were mediated by either stage or histotype. Each of the other dichotomous variables, including age, smoking history, grade, stage and node status, was similarly tested for its impact on prognosis by Cox regression analysis. The median p53 cut-off was also used in survival curves, examining the relapse or death rates of the p53-negative and p53-positive patients, which were plotted by the method of Kaplan and Meier and compared using log-rank statistics.

RESULTS

Immunoassay of the 86 NSCLC tumour extracts revealed widely distributed p53 protein concentrations (mean = 6.28 ng/ml, standard deviation = 12.99 ng/ml, median = 0.56 ng/ml, minimum = 0.04 ng/ml, maximum = 70.69 ng/ml), all of which exceeded the assay detection limit. Adjustment of these results for the total amount of protein extracted in each case led to a similarly positively skewed, somewhat bimodal distribution (Fig. 1), which had a mean of 1.22 ng/mg and a standard deviation of 2.24 ng/mg (range 0.005–10.97 ng/mg). The 25th percentile, median and 75th percentile of this distribution were 0.044 ng/mg, 0.14 ng/mg and 1.29 ng/mg, respectively. Protein-adjusted p53 concentrations were not significantly correlated with patient age ($r_s = 0.14$, $p = 0.198$) or number of pack-years of cigarette smoking ($r_s = 0.14$, $p = 0.229$) nor were they significantly associated with these variables and others, including gender, disease stage, histologic grade, histologic type, lymph node metastasis and whether or not adjuvant therapy was given, in contingency tables in which p53-positivity status was based on a median cut-off point (Table I). The same analysis performed using the 25th and 75th percentiles of the p53 protein distribution as cut-off points, as well as Mann-Whitney and Kruskal-Wallis tests, also did not yield statistically significant associations (data not shown), perhaps as a consequence of the small size of the study population.

TABLE I – ASSOCIATIONS BETWEEN p53 STATUS[1] AND OTHER CLINICOPATHOLOGIC VARIABLES

Variable	p53 < median patients (%)	p53 ≥ median patients (%)	*p* value[2]
Sex			
Female	11 (39)	17 (61)	
Male	32 (55)	26 (45)	0.17
Age (years)			
<66	23 (60)	15 (40)	
≥66	20 (42)	28 (58)	0.08
Stage[3]			
I–II	35 (51)	33 (49)	
III–IV	8 (47)	9 (53)	0.75
Histologic grade[4]			
G1–G2	31 (54)	26 (46)	
G3	11 (44)	14 (56)	0.39
Histologic type[5]			
Adenocarcincoma	19 (44)	24 (56)	
Squamous cell carcinoma	20 (54)	17 (46)	
Other	4 (67)	2 (33)	0.47
Lymph node metastasis			
No	26 (48)	28 (52)	
Yes	17 (53)	15 (47)	0.66
Smoking history[6]			
No	6 (75)	2 (25)	
Yes	34 (50)	34 (50)	0.18
Post-operative treatment[7]			
Not treated	28 (53)	25 (47)	
Treated	15 (45)	18 (55)	0.51
Recurrence			
No	27 (59)	19 (41)	
Yes	16 (40)	24 (60)	0.08
Death			
No	35 (55)	29 (45)	
Yes	8 (36)	14 (64)	0.14

[1]p53 expression status based on median cut-off level of 0.14 ng/mg.–[2]*p* values calculated from χ^2 tests.–[3]Pathologic stage unknown for 1 patient.–[4]Histologic grade unknown for 4 patients.–[5]Other tumours were of large cell and adenosquamous histotypes.–[6]Smoking history unknown for 10 patients.–[7]Included treatment with chemotherapy or radiotherapy or both.

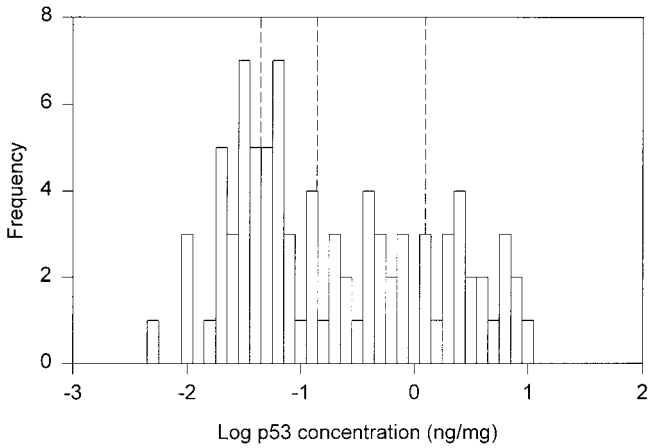

FIGURE 1 – Frequency distribution of logarithmically transformed p53 protein concentrations in the 86 non-small cell lung tumour extracts. From left to right, dashed lines indicate the 25th, 50th and 75th percentiles of the distribution.

The relationships between p53 protein concentration and patient disease-free survival and overall survival were initially examined without considering the variable follow-up times, with which p53 is negatively correlated ($r_s = –0.22$, $p = 0.039$). Levels of p53 protein were thus also found to be negatively correlated with the length of the time interval from surgery to first recurrence ($r_s = –0.36$, $p = 0.023$) but not with those leading to patient death ($r_s = –0.11$, $p = 0.586$). A stronger association between p53 protein status, defined by a median cut-off point, and patient relapse was also suggested in contingency tables (Table I), though the tendency to have had p53-positive tumours of patients who relapsed or of those who subsequently died did not reach statistical significance. Selection of other quartiles as cut-off points in the contingency tables similarly did not lead to significant *p* values (data not shown). Univariate Cox regression analysis, however, in which p53 concentrations were used as a 4-level categorical variable or a dichotomous variable, revealed that increasing amounts of p53 protein in lung tumour extracts were associated with progressively reduced disease-free and overall survival probabilities (Table II). Although the use of p53 as a continuous variable in the regression analysis showed trends suggesting a dose-response relationship between p53 and risk of relapse (HR = 1.01, $p = 0.097$) and death (HR = 1.01, $p = 0.050$), these relationships were also evident from the analysis presented in the table in which patients were divided into 4 groups by the 3 quartiles of the frequency distribution of p53

TABLE II – ASSOCIATIONS BETWEEN p53 STATUS AND DISEASE-FREE AND OVERALL SURVIVAL

p53 status	Disease-free survival			Overall survival		
	HR[1]	95% CI[2]	*p* value	HR[1]	95% CI[2]	*p* value
Univariate analysis (n = 86)						
Based on quartiles[3]						
1st quartile	1.00			1.00		
2nd quartile	0.93	0.35–2.48		0.83	0.20–3.37	
3rd quartile	2.05	0.83–5.06		1.55	0.41–5.76	
4th quartile	2.09	0.85–5.19		3.31	1.00–10.97	
p value for trend			0.025			0.021
Based on median value						
Negative	1.00			1.00		
Positive	2.19	1.15–4.15	0.016	2.47	1.03–5.94	0.043
Multivariate analysis[4] (n = 75)						
Based on quartile[3]						
1st quartile	1.00			1.00		
2nd quartile	1.27	0.47–3.48		0.97	0.21–4.45	
3rd quartile	3.74	1.25–11.17		2.16	0.51–9.18	
4th quartile	4.00	1.18–13.53		17.16	2.12–139.14	
p value for trend			0.010			0.016
Based on median value						
Negative	1.00			1.00		
Positive	3.02	1.50–6.06	0.002	3.72	1.44–9.57	0.007

[1]HR is hazard ratio estimated by the Cox proportional hazards regression model.–[2]CI is the confidence interval of the estimated HR.–[3]Estimated HR for second, third and fourth quartiles relative to first quartile are given.–[4]Included in the multivariate models were patient age, stage, histologic grade, lymph node status and smoking history.

protein concentrations. The simpler division of patients into p53-negative and p53-positive groups of equal size by merging the 2 lower and 2 upper quartile-based groups demonstrated over 2-fold increased risks of relapse and death in p53-positive compared to p53-negative patients. These differences in survival rate between the median-dichotomised p53-negative and p53-positive patients were also shown by Kaplan-Meier survival plots (Fig. 2). Adjustment for the effects of age, stage, grade, node status and smoking history in multivariate Cox models of disease-free and overall survival revealed that p53 protein concentrations elevated above the median indicated significantly reduced survival independently of these other factors (Table II). Inclusion of treatment in the same multivariate models resulted in p53 losing its significance for overall survival (RR = 2.33, $p = 0.12$) but not for disease-free survival (RR = 2.80, $p = 0.009$). The magnitude of the increased risk of relapse or death conferred by p53-positivity in multivariate analysis was comparable to that associated with stage III–IV disease but of less statistical significance than the risks associated with lymph node metastases (Table III). In contrast, patient age, smoking history and histologic grade dichotomised as above were of no prognostic value in our series of 86 patients.

Since the relationships demonstrated between p53-positivity and shortened disease-free and overall survival may have been restricted to patients whose tumours differed with respect to histologic type or anatomic extent, the risk of each outcome associated with p53-positivity was examined separately in subgroups defined by histotype and stage (Table IV). For patients with adenocarcinoma of the lung, the increased hazards of relapse and death estimated from the models for individuals with p53-positive tumour extracts were not significantly different from those for p53-negative patients. However, the more than 4-fold elevated risks of both end points for patients with p53-positive squamous cell carcinomas were of greater statistical significance. Although p53-positivity did confer an increased likelihood of relapse and death for both early-stage (I–II) and late-stage (III–IV) patients, the results were of greater statistical significance in early-stage lung cancer. In the more homogeneous subgroup of patients with stage I disease (n = 52), other trends similarly suggested that p53-positivity was associated with increased risk of relapse (HR = 2.21, $p = 0.12$) and death (HR = 4.33, $p = 0.19$). In patients with squamous cell carcinoma or early-stage disease (Table IV), but not in those with adenocarcinoma or in stages III or IV (data not shown), p53-positivity remained an indicator of unfavourable prognosis in multivariate regression models. These models, which adjusted the relationships between p53 and survival outcomes for the influences of age, stage and lymph node status, revealed that patients with p53-positive squamous cell tumours had an 11-fold increased risk for relapse and a 35-fold increased risk for death compared with p53-negative patients with the same histologic type. The greater effect of p53 upon survival in squamous cell carcinoma patients compared with patients with adenocarcinoma could not likely be explained by differences in power since there were no marked differences in the numbers and proportions of patients relapsing or dying between the 2 subgroups: 21 and 14 of 43 adenocarcinoma patients relapsed and died, respectively, whereas 16 and 8 of 37 squamous cell carcinoma patients relapsed and died, respectively. However, the results of formal tests of interaction did not support a difference in the prognostic value of p53 between the 2 histologic classifications since the p53-by-histotype interaction term was not statistically significant for either relapse ($p = 0.23$) or death ($p = 0.83$). p53-by-stage interaction was similarly not significant in univariate Cox models.

DISCUSSION

Point mutations and allelic loss of the *p53* gene are among the most common genetic lesions described in NSCLC, exceeded only by deletions on chromosome 3p (Brauch *et al*., 1987). Abundant evidence has suggested that this relatively high mutation rate results from the selective growth of tumour cell clones in which *p53* has been functionally inactivated (Sozzi *et al*., 1995). Since *p53* mutation appears to be an early, although not necessarily an initiating or even a required, event in lung tumorigenesis (Sozzi *et al*., 1992; Bennett *et al*., 1993; Fontanini *et al*., 1994), it may lead to a tumour phenotype which is unrelated to the local or systemic spread of the disease, represented by the stage classification. Pathologic stage is currently the major determinant for the receipt of adjuvant chemotherapy or radiotherapy; patients in early-stage disease are usually spared such treatment. The need for supplementary prognostic factors in NSCLC arises from the large proportion of patients with early-stage tumours, especially those without

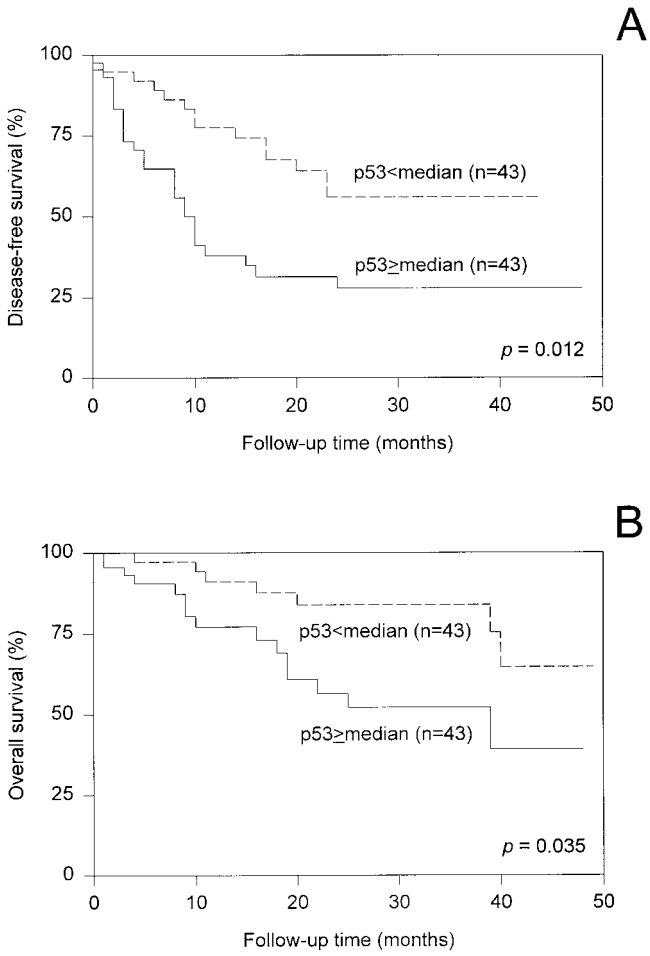

FIGURE 2 – Kaplan-Meier analysis of disease-free survival *(a)* and overall survival *(b)* of the 86 non-small cell lung carcinoma patients using the median value as the cut-off point for p53-positivity. *p* values were calculated by log-rank tests.

regional lymph node involvement, who nevertheless relapse and for whom aggressive adjuvant treatment may be warranted (Mountain, 1986). While a number of other morphologic and molecular features of NSCLC have been proposed to provide accurate prognostic information on which to base treatment decisions, none has emerged as unequivocally more valuable than stage alone. Some studies have shown mutation of the *p53* gene (Horio *et al*., 1993; Mitsudomi *et al*., 1993) or over-expression of the p53 protein (Quinlan *et al*., 1992) to be indicative of shorter survival of NSCLC patients, but others have been unable to demonstrate such associations (McLaren *et al*., 1992; Lee *at al*., 1995; Passlick *et al*., 1995; Nishio *et al*., 1996). Differences in statistical power resulting from variable population sizes, as well as inherent differences in the populations themselves, may underlie many of these discordant findings. However, also contributing to the lack of consensus among these studies may be the differing sensitivities and specificities of the methods used to ascertain *p53* mutational status.

The immunohistochemical detection of p53 protein accumulation in the nuclei of tumour cells, including those from NSCLCs, has generally been accepted as evidence of *p53* gene mutation. It has been estimated, however, that approximately 10–20% of *p53* point mutations occur outside the most frequently mutated exons 5 to 8 and are not accompanied by p53 protein over-expression (Casey *et al.,* 1996). Non-sense mutations, as well as nucleotide deletions and insertions, similarly are not associated with stabilisation of p53 protein, which may also occur by non-mutational mechanisms. Given the imperfect concordance between genetic alterations of *p53* and accumulation of its protein product and the relative insensitivity of indirect techniques of screening for *p53* mutations, such as single-strand conformation polymorphism analysis, it has been suggested that complete sequencing of all 11 exons is required to detect all *p53* sequence changes (Casey *et al.,* 1996). *In vitro* functional assays of *p53* cloned from tumour cells to assess the ability of *p53* mutants to regulate gene transcription in a sequence-specific manner, though biologically relevant, remain inappropriate for prognostic studies involving large numbers of patients. In contrast, immunohistochemical methods of detecting p53 protein have been predominantly used to demonstrate associations between *p53* abnormalities and survival of cancer patients. Compared to conventional immunostaining techniques, however, densitometric image analysis of stained tissues (Charpin *et al*., 1996) and ELISA-type analyses of tissue extracts (Vojtěšek *et al*., 1992; Hassapoglidou *et al*., 1993; Levesque *et al*., 1995; Thomas *et al*., 1997) may offer improved reproducibility. In addition, immunoassays, in their most common configuration, may possess greater specificity due to their use of 2 p53-directed antibodies. Despite these potential advantages, the use of ELISAs of p53 protein has been largely restricted to serum analysis (Luo *et al*., 1994), possibly reflecting the firm establishment of p53 immunostaining as a research tool in many histology laboratories, the inability of ELISAs to localize p53 accumulation in the context of histologic features and the requirement of fresh-frozen tissue specimens. General concordance between the findings of immunoassay and immunohistochemical analyses of several tissues has been reported (Vojtěšek *et al*., 1992). In a study of 91 lung carcinomas of various histologic types, we found a strong correlation ($r_s = 0.65$, $p < 0.001$) between the p53 protein concentrations determined by our immunofluorometric assay in extracts of fresh-frozen tissues and the immunohistochemical scores reflecting the proportion of stained malignant cells, intensity of staining and tumour cellularity of matched formalin-fixed, paraffin-embedded tissues (Levesque *et al*., 1997). Since there were cases in that study for which the 2 methods were in disagreement, we considered it possible that p53 ELISA might yield prognostic information different from that of p53 immunostaining. The present study includes 82 NSCLC specimens assessed previously by the 2 methods and utilizes essentially the same median cut-off point for p53-positivity, equal to 0.13 ng/mg and 0.14 ng/mg in the previous and present studies, respectively. Selection of the median value as the cut-off was arbitrary but reasonable given the bimodal frequency distribution of p53 protein concentrations (Fig. 1) and consistent with the range of p53-positivity rates (40–80%) reported by others (McLaren *et al.,* 1992; Bennett *et al.,* 1993; Fontanini *et al*., 1994; Harpole *et al.,* 1995; Lee *et al.,* 1995; Passlick *et al.,* 1995; Top *et al.,* 1995; Nishio *et al.,* 1996). Furthermore, we have shown that a small number of histologically normal lung tissues displayed p53 contents no greater than 0.07 ng/mg (Levesque *et al.,* 1997).

Using a commercially available pantropic p53 ELISA kit (Oncogene Research), Pappot *et al*. (1996) did not find a statistically significant difference in overall survival of 228 NSCLC patients whose tumour extracts had p53 levels below *vs.* above the median cut-off point. These workers observed, however, significantly higher p53 protein concentrations in squamous cell carcinomas than in adenocarcinomas, though the prognostic values of p53 within these subgroups were similiar, as well as higher p53 levels in patients who were older and who had large, late-stage tumours. Several other studies have also shown a slightly greater percentage of squamous cell carcinomas to exhibit p53 immunostaining than adenocarcinomas (Mitsudomi *et al*., 1993; Lee *et al*., 1995; Nishio *et al*., 1996), a relationship not consistent with our finding of a trend suggesting higher p53 levels in adenocarcinomas compared to squamous cell carcinomas. This latter relationship, however, was not statistically significant and should be confirmed in further studies of ELISA-detected p53 protein and NSCLC patient progno-

TABLE III – ASSOCIATIONS BETWEEN STATUS OF OTHER CLINICO-PATHOLOGIC VARIABLES AND PATIENT SURVIVAL

Variable status	Disease-free survival			Overall survival		
	HR[1]	95% CI[2]	*p* value	HR[1]	95% CI[2]	*p* value
Univariate analysis (n = 86)						
Age (years)						
≥66 *vs.* <66	1.17	0.63–2.19	0.61	0.79	0.34–1.86	0.59
Smoking history[3]						
Yes *vs.* no	0.81	0.32–2.07	0.65	0.67	0.19–2.33	0.53
Histologic grade[4]						
G3 *vs.* G1–G2	1.15	0.57–2.32	0.69	1.40	0.57–3.45	0.46
Stage[5]						
III–IV *vs.* I–II	2.63	1.29–5.39	0.008	3.80	1.56–9.26	0.003
Lymph node status						
Positive *vs.* negative	3.10	1.65–5.83	<0.001	6.63	2.55–17.22	<0.001
Multivariate analysis (n = 75)						
Stage[5]						
III–IV *vs.* I–II	3.18	1.41–7.17	0.005	4.45	1.55–12.81	0.006
Lymph node status[6]						
Positive *vs.* negative	4.32	2.11–8.83	<0.001	10.68	3.50–32.59	<0.001

[1]HR is hazard ratio estimated by the Cox proportional hazards regression model.–[2]CI is the confidence interval of the estimated NR.–[3]Smoking history unknown for 10 patients.–[4]Histologic grade unknown for 4 patients.–[5]Pathological stage unknown for 1 patient.–[6]Multivariate models were adjusted for age, smoking history and p53 status.

TABLE IV – ASSOCIATIONS BETWEEN p53 STATUS[1] AND SURVIVAL IN PATIENT SUBGROUPS

p53 status	Disease-free survival			Overall survival		
	HR[2]	95% CI[3]	*p* value	HR[2]	95% CI[3]	*p* value
Univariate analysis						
Adenocarcinoma patients (n = 43)						
Positive *vs.* negative	1.62	0.68–3.88	0.280	2.07	0.69–6.21	0.20
Squamous cell carcinoma patients (n = 37)						
Positive *vs.* negative	4.41	1.40–13.88	0.011	4.64	0.91–23.73	0.066
Patients in stage I or II (n = 68)						
Positive *vs.* negative	3.02	1.36–6.72	0.007	3.05	0.93–10.02	0.066
Patients in stage III or IV (n = 17)						
Positive *vs.* negative	2.97	0.69–12.77	0.14	4.42	0.81–23.97	0.085
Multivariate analysis						
Squamous cell carcinoma patients (n = 37)[4]						
Positive *vs.* negative	11.68	2.89–47.20	<0.001	35.69	2.85–447.86	0.006
Patients in stage I or II (n = 68)[5]						
Positive *vs.* negative	4.82	1.98–11.76	<0.001	5.11	1.43–18.21	0.012

[1]p53 expression status based on median cut-off level of 0.14 ng/mg.–[2]HR is hazard ratio estimated by the Cox proportional hazards regression model.–[3]CI is the confidence interval of the estimated HR.–[4]Multivariate models were adjusted for patient age, stage and lymph node status.–[5]Mulitvariate models were adjusted for patient age and lymph node status.

sis. Although a few studies have reported p53 accumulation in NSCLC to be unrelated to any other clinico-pathologic variable (Chiba *et al*., 1990), a relationship between *p53* abnormalities and lifetime exposure to cigarette smoke has been a finding shared by a large number of studies (Suzuki *et al*., 1992; Nishio *et al*., 1996). In survival analysis, levels of p53 protein exceeding the median of our series of 86 extracts of NSCLC tissues were also associated with reduced disease-free and overall survival. Moreover, we presented evidence that progressively increasing p53 concentrations may be associated with correspondingly increasing risks of relapse or death. That our findings differed from those reported by the only other study of the prognostic utility of ELISA-detected p53 protein in NSCLC (Pappot *et al*., 1996) was surprising since the median p53 concentration (0.10 ng/mg) used in the latter study was comparable to our median value and the median ages and proportions of patients in the various stages and histologic types were similiar, though the duration of follow-up for the larger number of patients in that study was greater. Other factors possibly accounting for the different conclusions of the 2 studies might also be differences in post-operative treatment or other undefined demographic or clinical differences between the patient populations which might have modified the impact of p53 on survival. Since our series of NSCLC patients was not adequately characterised in terms of post-operative chemotherapy and radiotherapy administration, further studies of larger patient samples with detailed adjuvant treatment profiles are needed to determine the prognostic impact of ELISA-determined p53 protein in patients receiving different post-operative treatments.

In multivariate analysis, the increased risk of relapse and death associated with p53-positivity was also shown to be of much greater magnitude in patients with squamous cell carcinomas than in those with adenocarcinomas. Previous studies had also suggested interaction between histologic type and p53 accumulation status but in such a way that it was in adenocarcinoma patients and not in those with squamous cell carcinoma that p53 was a statistically significant prognostic indicator (Nishio *et al*., 1996; Fukuyama *et al*., 1997). Taken together with our finding of a slight trend of greater p53-positivity in adenocarcinomas, the direction of the subgroup differences in the prognostic effect of p53 was quite surprising. Statistically significant interaction between p53 and histotype could not be demonstrated in Cox models of either relapse or death, and it remains possible that differences between patients with adenocarcinoma and squamous cell carcinoma which

were not included in the multivariate models might have had a confounding influence on the relationships between p53 and survival. In particular, differences between subgroups in terms of chemotherapeutic agents received post-operatively could have led to our findings. Although power calculations were not performed, the similar numbers of patients and events within the 2 subgroups did not imply differences in power as an explanation. In contrast to the apparent differences with respect to p53 prognostic value between the 2 most common NSCLC histologic groups, patients with p53-positive tumours had similarly elevated risks for both outcome events irrespective of whether they had early (I–II)- or late (III–IV)-stage disease. Only those in the early-stage group were found in multivariate analysis to relapse or die at rates related to p53 status, but this finding is likely due more to the small number of patients in the late-stage group than to any true interaction between p53 status and stage. When the analysis was restricted to patients in stage I, who constituted 60% of the cohort, the same trends for poor survival associated with p53-positivity were evident. Other workers have also demonstrated *p53* alteration to be indicative of poor prognosis in stage I or II NSCLC (Quinlan *et al*., 1992; Harpole *et al*., 1995) but not in stages III or IV (Passlick *et al*., 1995), while Mitsudomi *et al*. (1993) found p53 to be related to survival only in late-stage patients. The lack of consensus evidently points to a need for further investigation of larger, well-characterised patient series assessed for *p53* abnormalities by reproducible techniques.

In this study, we have shown that the quantitative assay of p53 protein by a simple ELISA applied to extracts prepared from surgically resected NSCLCs may identify patients at increased risk of unfavourable outcome. We have also provided evidence that the utility of p53 quantification may be particularly relevant for the prognosis of patients with squamous cell carcinoma of the lung. Since our data confirm a number of previous reports of the prognostic value of p53 over-expression in this disease, we propose that the detection of p53 protein by ELISA may serve as an alternative to the more commonly employed immunohistochemistry. Given the small size and heterogeneity of our sample of patients, however, further studies are needed to unequivocally demonstrate the prognostic value of ELISA-detected p53 protein accumulation in patients with NSCLC.

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