Pergamon

European Journal of Cancer, Vol. 34, No. 9, pp. 1352–1357, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(98)00067-7

Original Paper

Immunohistochemically Detected p53 and P-glycoprotein Predict the Response to Chemotherapy in Lung Cancer

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While resistance to chemotherapy is a major problem in lung cancer treatment, there is no useful predictor of treatment response. We thus designed this study to determine the utility of p53 and P-glycoprotein expression in predicting the response to chemotherapy in patients with primary lung cancer, retrospectively. We evaluated transbronchial biopsy (TBB) specimens from 60 patients with lung cancer, who were previously untreated. Formalin-fixed, paraffin-embedded TBB specimens were immunostained using anti-p53 antibody (DO-1) and anti-P-glycoprotein antibody (JSB-1). The positivity of p53 was 63%, and that of P-glycoprotein was 17%. No correlation was observed between p53 and P-glycoprotein immunostaining. Positivity of p53 correlated significantly ($P = 0.004$) with a lack of response to chemotherapy in non-small cell lung cancer (NSCLC), but not in small cell lung cancer (SCLC). In contrast, positivity of P-glycoprotein was correlated with chemotherapy resistance in SCLC ($P = 0.003$), but not in NSCLC. Multiple logistic regression analysis revealed that positive immunostaining for p53 was a significant risk factor for chemotherapy resistance in NSCLC. These results suggest that immunostaining of p53 and P-glycoprotein for TBB specimens may help to predict response to chemotherapy in NSCLC and SCLC, although the results should be confirmed in a larger, more homogeneous series. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: p53, P-glycoprotein, lung cancer, chemotherapy, immunohistochemistry
Eur J Cancer, Vol. 34, No. 9, pp. 1352–1357, 1998

INTRODUCTION

LUNG CANCER is a major cause of cancer-related deaths worldwide [1]. This disease is divided morphologically into two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC is extremely aggressive, but is initially highly sensitive to both chemotherapy and radiotherapy. Therefore, treatment mainly consists of these modalities. In contrast, NSCLC, which comprises more than 75% of lung tumours, is generally resistant to chemotherapy, and, as a result, surgery is the treatment of choice for localised disease. However, approximately 70% of NSCLC cases are at an advanced stage at diagnosis and thus are treated with chemotherapy, but the response rate is low and the beneficial effect on survival remains controversial [2]. Although chemotherapy is often used in treating patients with both NSCLC and SCLC, the difference in chemosensitivity for both types of cancer has yet to be elucidated.

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Received 29 Sep. 1997; revised 26 Jan. 1998; accepted 11 Feb. 1998.

A mutation of the *p53* gene is one of the most common abnormalities found in all types of human tumours [3] and has been reported in more than 50% of lung tumour samples [4, 5]. Clinically, some studies have linked either a *p53* gene mutation or p53 overexpression to aggressive tumour behaviour and a poor prognosis [6, 7], but others have reported no such association [8, 9]. Lowe and colleagues [10] demonstrated *in vitro* that cells lacking wild-type *p53* were resistant to both ionising radiation and cancer chemotherapy, whereas cells that expressed wild-type p53 were sensitive, and exhibited cell death by apoptosis. The therapeutic effect of several antineoplastic agents may thus be mediated through DNA damage and the secondary induction of apoptosis [11, 12]. In general, mutant *p53* suppresses the function of wild-type p53, which then arrests the cell cycle at the G1 phase during DNA repair [13, 14]. These observations suggest that *p53* mutations may potentially provide a genetic basis for drug resistance [15–17]. However, few clinical data have been reported to support this idea [18]. Recently, Rusch and associates [19] reported that an aberrant p53 expression

correlated with resistance to cisplatin-based chemotherapy in NSCLC.

Alternatively, P-glycoprotein is a membrane transport protein (energy-dependent efflux pump) that is encoded by the multidrug resistance gene (*MDR1*) of human cells [20]. Increased concentrations of P-glycoprotein or its RNA transcript are found in tumours of epithelial, neurogenic, mesenchymal and haematopoietic origins [21–23]. High tissue levels of P-glycoprotein can predict the success or failure of chemotherapy in some patients with neuroblastoma, ovarian cancer and SCLC [24, 25].

Transbronchial biopsy (TBB) is a very useful technique for obtaining tumour specimens before therapy [26]. In this study, we analysed the relationship between p53 and P-glycoprotein overexpression and the patient's response to chemotherapy, using TBB specimens.

PATIENTS AND METHODS

Patient population

Between April 1991 and March 1994, we enrolled 60 sequential previously untreated patients, with histopathologically confirmed (in TBBs) primary lung cancer who were also treated with chemotherapy at Kyushu University Hospital. All had bidimensionally measurable lesions on chest X-ray or computed tomographic (CT) scan. The pretreatment evaluation for staging consisted of a chest X-ray, bone scan, a CT scan of the chest, abdomen and brain. The clinical stage of disease was defined using the current International Staging System [27].

The patients included 42 males and 18 females, ranging in age from 39 to 82 years (mean 61 years). Thirty tumours were diagnosed as SCLC and 30 as NSCLC, including 21 adenocarcinomas, six squamous cell carcinomas and three large cell carcinomas.

Chemotherapy and response criteria

The chemotherapy regimens are summarised in Table 1. 20 patients with NSCLC received a combination of cisplatin, carboplatin and vindesine (PCV). The other 10 patients with NSCLC received cisplatin plus vindesine (PV). Patients with SCLC received one of the following combination regimens: cisplatin, carboplatin and etoposide (PCE; 11 patients); carboplatin plus etoposide (CE; 8 patients); cyclophosphamide, doxorubicin and vincristine (CAV; 7 patients); and cisplatin plus etoposide (PE; 4 patients). All patients received two or more courses of chemotherapy at 3–4 week intervals. We used the standard response criteria [28] for evaluating the patient's response. An assessment of the tumour response was performed after every course by repeating appropriate radiographic studies. The response rate was defined as the number of cases having a complete response plus those having a partial response divided by the total number of patients.

Immunohistochemistry

We used the immunostaining method of Shin and colleagues [29] to enhance immunoreactivity in formalin-fixed TBB samples. Briefly, 5 μm sections were cut from paraffin blocks and then allowed to air dry. The slides were deparaffinised in xylene and absolute alcohol, and were autoclaved at 121°C with distilled water for 20 min. After cooling, 10% rabbit serum was placed on the slides to reduce any background staining. The slides were then incubated overnight with the primary antibody at 4°C in a moist chamber. The primary anti-p53 monoclonal antibody was DO-1 (Ab-6; Oncogene Science, New York, U.S.A.) which recognises both the wild-type and the mutant forms of p53. A dilution of 1:200 (0.5 μg/ml) was used. JSB-1 (Nichirei Co., Japan), an anti-P-glycoprotein that reacts with human P-glycoprotein, was used at a dilution of 1:200 (0.75 μg/ml). Non-specific mouse IgG was used as the negative control. After washing with phosphate-buffered saline (PBS), the slides were incubated with secondary antibody for 30 min (biotinylated anti-mouse IgG; Nichirei). After washing with PBS, the slides were then incubated with streptavidin–peroxidase reagent (Nichirei) for 30 min. After another PBS wash, the antigen–antibody complex was visualised using a 0.05% solution of diaminobenzidine tetrahydrochloride in PBS for 5 min. All sections were evaluated by two observers (including one pathologist) without any knowledge of the clinical outcome, comparing immunostained samples with haematoxylin–eosin slides. At least 500 cancer cells were counted. Sections of human lung cancer tissue known to be positive for p53 protein served as positive controls for p53 staining. A tumour was considered to be immunopositive for p53 when the number of cancer cells with stained nuclei exceeded 10%. It has been reported that the best concordance (90%) between immunohistological and molecular biological analyses has been observed using this cut-off value [9]. Immunostaining for P-glycoprotein was defined as positive when the plasma membrane was stained linearly on the cell membrane, compared with the staining of the human adrenal gland (the positive control). A tumour sample was evaluated as P-glycoprotein negative when none of the tumour cells demonstrated immunostaining [30].

Statistical analysis

The chi-squared test or Fisher's exact test were used to evaluate the association between immunohistochemical

Table 1. *Chemotherapy regimens*

Histology		Drug	Dose (mg/m^2)	Schedule (day)
NSCLC	PCV			
		Cisplatin	80	1
		Carboplatin	100	2, 3, 8
		Vindesine	2	2, 3, 8
	PV			
		Cisplatin	80	1
		Vindesine	3	1, 8, 15
SCLC	PCE			
		Cisplatin	80	1
		Carboplatin	100	2, 3, 8
		Etoposide	50	1, 2, 3, 8
	CE			
		Carboplatin	300	1
		Etoposide	100	1, 2, 3
	CAV			
		Cyclophosphamide	750	1
		Doxorubicin	50	1
		Vincristine	1.5	1
	PE			
		Cisplatin	80	1
		Etoposide	100	1, 2, 3

All drugs were administered intravenously.

expression and the clinical variables. Multivariate analysis was performed with the PC/SAS statistical package (SAS Institute). A logistic regression analysis was used to control possible confounding factors and to estimate the odds ratio. All reported P values are two-sided. A level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of chemotherapy

As described in 'Patients and Methods', 30 patients with NSCLC received platinum-based chemotherapy, while 30 patients with SCLC received either platinum-based chemotherapy or a CAV regimen. Partial and complete responses were achieved in two or less courses of chemotherapy. The overall response for patients with NSCLC was 33% (10/30; 7/20 for PCV and 3/10 for PV). The overall response for patients with SCLC was 73% (22/30; 9/11 for PCE, 5/8 for PE, 4/4 for PE and 4/7 for CAV). Although the chemotherapy regimens differed in their composition and intensities, there was no significant difference in their response rate.

Immunostaining for p53 and P-glycoprotein

Figure 1 demonstrates the staining pattern of p53 and P-glycoprotein. p53 overexpression was detected only in the nuclei, and P-glycoprotein was stained on the cell membrane. Tumours from 38 of the 60 patients (63%) stained positively with the anti-p53 antibody. Positivity for P-glycoprotein was detected in 10 patients (17%). 7 patients (12%) showed positive staining for both p53 and P-glycoprotein. No correlation was observed between p53 and P-glycoprotein immunostaining. The immunostaining results of p53 and P-glycoprotein did not differ significantly according to sex, age, histology, clinical stage or performance score (Table 2).

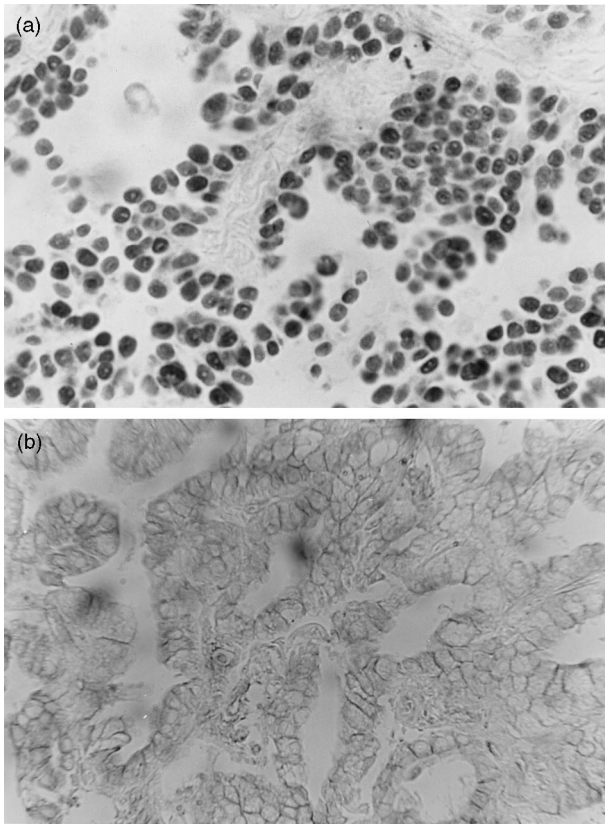

Figure 1. Immunohistochemical demonstration of p53 and P-glycoprotein. (a) p53 (DO-1); the nuclei of adenocarcinoma cells show abnormally high concentrations of p53 protein. (b) P-glycoprotein; adenocarcinoma of the lung showing a strong ring-shaped JSB-1 immunoreactivity in the tumour cells.

Table 2. Results of immunostaining

Characteristics	p53+ (%)	P*	P-glycoprotein+ (%)	P†
Overall	38/60 (63)		10/60 (17)	
Sex				
Male	27/42 (64)	0.999	4/42 (10)	0.052
Female	11/18 (61)		6/18 (33)	
Age (years)				
< 60	16/24 (67)	0.870	6/24 (25)	0.178
≥ 60	22/36 (61)		4/36 (11)	
Histology				
NSCLC	18/30 (60)	0.789‡	6/30 (20)	0.514‡
Adenocarcinoma	15/21 (71)		5/21 (24)	
Squamous cell carcinoma	3/6 (50)		0/6 (0)	
Large cell carcinoma	0/3 (0)		1/3 (33)	
SCLC	20/30 (67)		4/30 (13)	
Stage				
II, III	12/21 (57)	0.653	5/21 (24)	0.467
IV	26/39 (67)		5/39 (13)	
Performance score				
0, 1	25/43 (58)	0.241	7/43 (16)	0.999
2, 3	13/17 (76)		3/17 (18)	

NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer. *P value obtained based on the chi-squared test. †P value based on Fisher's exact test. ‡NSCLC versus SCLC.

Response to chemotherapy according to immunostaining

The relationship between immunostaining and the response to chemotherapy is summarised in Tables 3 and 4. In NSCLC, the p53 negative group responded to chemotherapy significantly better than the p53 positive group: with a response rate of 67% versus 11% ($P = 0.004$). P-glycoprotein seemed to confer a slight resistance to chemotherapy in the patients with NSCLC, but this was not statistically significant, ($P = 0.633$). In patients with SCLC, the immunopositivity for P-glycoprotein, but not for p53, correlated significantly with a lack of response to chemotherapy (0% versus 85%, $P = 0.003$). In SCLC, the P-glycoprotein positive group consisted of only 4 patients. The characteristics of these patients were not specific according to performance status, clinical stage and age. Similar results were obtained when the relationship between the staining results and the clinical response was evaluated in terms of the predominant chemotherapy regimens. The response rate of the PVC regimen for NSCLC ($n = 20$) was 9% in the p53 positive group and 67% in the p53 negative group ($P = 0.027$), but P-glycoprotein immunostaining had no effect on the response to chemotherapy. In the PCE regimen for SCLC ($n = 11$), only staining for P-glycoprotein correlated with the response, with a response rate of 0% for the P-glycoprotein positive group and 90% for the negative group ($P = 0.012$). Progressive disease was observed in 7 patients (6 patients with NSCLC and 1 with SCLC), and 5 of the 6 patients with NSCLC (83%) demonstrated p53 positive and P-glycoprotein negative immunostaining. In the multiple logistic regression analysis, only p53 was shown to be a statistically significant predictor of chemotherapy resistance in NSCLC (Table 5). However, the data on SCLC could not be analysed by multiple logistic regression analysis because there was no responder in the P-glycoprotein positive group. The multivariate analysis on SCLC without data of P-glycoprotein showed no significant predictors of response to chemotherapy (data not shown).

Table 3. Response to chemotherapy according to immunostaining: non-small cell lung cancer

Immunostaining	CR	PR	SD	PD	Response rate	*P* value*
p53 +	0	2	11	5	11% (2/18)	0.004
p53 –	0	8	3	1	67% (8/12)	
P-glycoprotein +	0	1	5	0	17% (1/6)	0.633
P-glycoprotein –	0	9	9	6	38% (9/24)	

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. *Fisher's exact test.

Table 4. Response to chemotherapy according to immunostaining: small cell lung cancer

Immunostaining	CR	PR	SD	PD	Response rate	*P* value*
p53 +	3	12	4	1	75% (15/20)	0.999
p53 –	0	7	3	0	70% (7/10)	
P-glycoprotein +	0	0	3	1	0% (0/4)	0.003
P-glycoprotein –	3	19	4	0	85% (22/26)	

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease. *Fisher's exact test.

Table 5. Multiple logistic regression analysis for chemotherapy response: non-small cell lung cancer

Factors	Odds ratio (95% CI)
Age (years)	
60 ≥ versus 60 <	3.42 (0.19–60.77)
Gender	
Male versus female	2.01 (0.13–31.96)
Performance score	
2, 3, 4 versus 0, 1	1.65 (0.06–42.44)
Stage	
IV versus III	1.94 (0.11–34.33)
P-glycoprotein	
+ versus –	10.28 (0.32–333.64)
p53	
+ versus –	20.50 (1.68–250.95)*

95% CI, 95% confidence interval. *$P < 0.05$.

DISCUSSION

The chemoresistance of malignancies such as NSCLC continues to present a major problem. Improved techniques are thus needed to identify chemosensitivity and many *in vitro* and *in vivo* chemosensitivity assays have been examined to predict the response to chemotherapy. However, the results of these assays have yet to reflect satisfactorily the response of a patient's tumour to chemotherapy [31]. Insight into the mechanism of drug resistance has progressed rapidly, and it has thus been determined that the chemoresistance of cancer cells is influenced by several factors [32].

Although TBB specimens are the best suited for the immunohistochemical prediction of chemosensitivity of lung cancer before therapy, few previous studies have used such specimens [33]. We studied the immunostaining of TBB specimens for P-glycoprotein and p53 based on the response to chemotherapy, while considering its usefulness and convenience.

P-glycoprotein is thought to play a major role in drug resistance. However, its clinical utility is uncertain [34], because the RNA transcript technique is complicated and immunohistochemical methods are not sufficiently sensitive. Toth and colleagues [30] recently developed a new staining method for detecting P-glycoprotein using JSB-1 antibody in formalin-fixed, paraffin-embedded human tissue specimens using an oven to enhance immunoreactivity. In their study, 2 of the 26 cases of NSCLC were positive for P-glycoprotein, and their results were consistent with the findings of other studies using frozen sections. By using hydrated autoclave pretreatment to enhance P-glycoprotein immunoreactivity, as originally reported by Shin and colleagues [29], we demonstrated that nine of 60 lung cancers were positive. Our findings were consistent with those of Toth and colleagues [30]. Immunopositivity for P-glycoprotein correlated significantly with a lack of response to chemotherapy in SCLC, but not in NSCLC. Holzmayer and associates [25] reported that the presence of MDR1-expressing tumour cells may be useful as a predictive marker for clinical resistance to combination chemotherapy in SCLC, using polymerase chain reaction (PCR). Our results were consistent with these. However, no such association was observed in NSCLC. In NSCLC, the number of P-glycoprotein positive cases was only 6. Thus, with a larger number of cases, immunostaining

of P-glycoprotein might also become a meaningful predictor of chemoresistance in NSCLC.

Although many of the primary cellular targets of many anticancer agents have been identified, little is known about the process that leads to the selective death of cancer cells. Recent findings on programmed cell death, apoptosis, have forced a reconsideration of the mechanism by which tumour cells acquire or lose their sensitivity to cytotoxic agents. Lowe and colleagues [10] reported that wild-type *p53* is required to activate apoptosis efficiently following the administration of radiation therapy or chemotherapy. Mutant *p53* is considered to suppress the function of wild-type *p53*, by what is called the 'dominant negative' effect, even if loss of heterozygosity of the wild-type *p53* allele exists. Thus, mutant *p53* may produce a resistance to chemotherapy of neoplastic tumours. More than 50% of cases of primary lung cancer have a genetic abnormality in *p53*, and most are missense mutations [3]. Mutant p53, which has a longer half-life than the wild type, can be detected immunohistochemically. Although the concordance between a *p53* gene mutation and the accumulation of p53 protein is not perfect, immunoreactivity does help indicate which tumours have an altered *p53* function. The criteria for defining the immunopositivity of p53 vary in the literature. We used the relatively common criterion in which the number of cancer cells with stained nuclei exceeded 10%. Recently, Nishio and associates [9] reported that an excellent concordance (90%) was obtained using a 10% cut-off value of p53-immunostaining between the immunohistological and molecular biological analyses.

We found that positive staining for p53 was significantly correlated with chemoresistance of NSCLC, but not with that of SCLC. Recently, Rusch and colleagues [19] reported that aberrant p53 expression correlated with resistance to cisplatin-based chemotherapy in NSCLC. Thus, p53 status was considered to be important for chemoresistance in NSCLC. Nevertheless, the association between p53 immunostaining and chemoresistance was considered to be useful in NSCLC for predicting the response to chemotherapy, and as a result, the administration of ineffective chemotherapy can be avoided.

Our results suggest that the mechanisms of chemoresistance may vary according to the type of tumour. In fact, recent reports have also suggested that p53 immunostaining may predict response to chemotherapy in breast cancer and NSCLC [18, 19], but not in ovarian cancer [35]. It has recently been reported that p53-independent apoptosis can be induced by some chemotherapeutic drugs, such as paclitaxel [36]. P-glycoprotein has been reported to be able to predict response in ovarian tumours, neuroblastoma and SCLC [24, 25]. Recently, many mechanisms of drug resistance have been reported, such as glutathione transferases, multidrug resistance-associated protein (MRP) and *HER-2/neu* gene expression [33, 37, 38]. Thus, in general, chemoresistance is considered to be complicated by different types of drug resistance.

In this retrospective analysis of TBB specimens from 60 patients, we found that positive immunostaining for p53 was a predictor of the response to chemotherapy in patients with NSCLC, while positive immunostaining for P-glycoprotein was a predictor of the response to chemotherapy in patients with SCLC. Our findings, therefore, indicate that immunostaining for p53 and P-glycoprotein in TBB samples from patients with lung cancer may be clinically useful in predicting resistance to chemotherapy. However, these results were obtained from a relatively small number of patients, while, in addition, the chemotherapy regimens were also heterogeneous. Thus, a prospective clinical study of a homogeneous patient series is needed to compare the therapeutic response of patients in terms of immunostaining of tumours for p53 and P-glycoprotein.

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Acknowledgement—The authors wish to thank Ms A. Nakashima for technical assistance.