Ras and p53 expression in non-small cell lung cancer patients: p53 over-expression correlates with a poor prognosis

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Abstract. Expression of the tumor suppressor gene p53 and the *ras* oncogene were examined in 46 tumor and nodal specimens of non-small cell lung cancer (NSCLC) using the antibodies p53 pAb 240 and *ras* Y13-259 respectively. p53 expression was elevated in 46% and *ras* p21 was over-expressed in 85% of the tumor specimens analyzed. Fifteen cases of benign lessions were also assessed for both *ras* p21 and p53 expression; all were found to have negative staining. p53 over-expression was found to correlate with a poor prognosis in both the tumor specimens ($p<0.05$) and in the nodal tissues ($p<0.005$). *Ras* p21 over-expression was found to be associated with survival ($p<0.1$) in both the tumor and the nodal specimens. Stage of the disease correlated with survival; similarly both p53 and *ras* p21 over-expression correlated with stage. No correlations were found with the pathological grade of the tumors nor with a history of smoking or duration of smoking. No K-*ras* mutations at codon 12 were observed in a further 15 NSCLC specimens analyzed. These results indicate that the p53 gene in particular plays a role in the stages of NSCLC.

Introduction

Lung cancer is the most frequently occurring fatal cancer in the western societies. Even with the best current approaches of early detection less than 10 per cent of all newly diagnosed patients will be cured. Non-small cell lung cancers (NSCLC) account for approximately 75 per cent of all lung cancers (1). Recent advances in molecular biology of lung cancer has provided a genetic framework in which the pathogenesis of the disease may be described (2,3). The evidence to date suggests that changes in dominant oncogenes and in tumor suppressor genes are most likely a prerequisite for malignant transformation (4-5). Mutation in the *ras* oncogene family and in the p53 tumor suppressor gene appear to be the most frequent genetic events in lung cancer (6-9). Mutations in the K-*ras* gene have been found by a number of research groups in NSCLC's (10-16) and K-*ras* mutations have been shown to be associated with a history of smoking in adenocarcinomas (6,7,10). The *ras* p21 protein has also been found to be over-expressed at a high frequency in NSCLC compared to SCLC (17,18). Evidence from both NSCLC cell lines and fresh tumor specimens has indicated that the K-*ras* gene is particularly implicated in the development of adenocarcinomas of the lung.

Approximately half of the adult cancers, including lung, breast, colon, esophagus, and skin cancers contain p53 mutations (19) and aberrant p53 expression is now considered to be one of the most common genetic features in a wide range of human cancers (20). p53 has recently been shown to have a biochemical role as a specific transcription factor and to have a biological role as a G1 checkpoint control for DNA damage (21-23).

A number of authors have described p53 mutations in lung carcinomas (8,9,24-26) and also over-expression of the p53 proteins (27-29). However, there is little information to date concerning the clinical outcome of lung cancer patients with regard to mutations or over-expression of the p53 tumor suppressor gene in both tumor and lymph node metastasis specimens.

In the current study we have evaluated both *ras* p21 and p53 expression in 46 NSCLC and analyzed this data with a range of clinico-pathological parameters, smoking history and survival.

Materials and methods

Patients and Pathology. Forty six specimens of surgically treated lung cancer patients and fifteen specimens of benign lung lesions from patients treated at Metaxa Anticancer Hospital in Pireas, Greece were analyzed in formalin fixed paraffin embedded sections. Tumors were classified according to the current World Health Organization typing of lung tumors (30) and the staging was done according to the new international staging system for lung cancer (31). No patient had received treatment of any type for their bronchial

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carcinomas. Pathology of the 46 surgical specimens showed that 20 were adenocarcinomas and 26 squamous cell carcinomas.

Immunohistochemical analysis. The following methods were employed. For the detection of *ras* p21 the ABC method was used. The rat monoclonal antibody Y13-259 recognizing the *ras* p21 proteins (32) was used for the immunohistochemical analysis of the *ras* p21 protein. Tissue sections 5μm thick were mounted on slides and deparaffinised. Endogenous peroxidase activity was blocked by immersing the sections for 30 min in an aqueous solution of 3% H_2O_2 in the dark. The sections were washed with PBS and treated with the Y13-259 rat monoclonal antibody, goat anti-rat IgG, streptavidin-peroxidase and DAB sequentially as previously described (33). Chinese hamster lung (CHL) cells, which express *ras* p21 at very low levels and the transformed cell line FHO5T1, which contains the mutated T24 H-*ras*1 oncogene inserted in a high expression vector (34) were used as controls.

Immunohistochemical analysis of the p53 protein was undertaken using the mouse anti-p53 monoclonal antibody pAb 240 (Ab-3 from Oncogene Science). pAb 240 does not bind to normal (wild type) p53 protein but recognizes a common conformational epitope on mutant p53 proteins which results from different activating mutations (35). The PAP method was used for the immunohistochemical analysis of the p53 protein. Tissue sections 5μm thick were mounted on slides and deparaffinised. Endogenous peroxidase activity was blocked by immersing the sections for 30 min in an aqueous solution of 3% H_2O_2 in the dark. The sections were washed with PBS and treated with the pAb 240 mouse anti-p53 monoclonal antibody dissolved in 5 volumes of PBS buffer and 5 volumes of bovine serum albumin 1% in ddH_2O for 1 h at 37°C in a humidified atmosphere. The slides were treated sequentially as follows: they were washed twice with PBS for 5 min each, treated with rabbit anti-mouse IgG conjugated with peroxidase 1:10 in 5 volumes PBS and 5 volumes normal human serum, incubated for 30 min at 37°C in a humidified atmosphere, washed twice with PBS for 5 min each, treated with swine anti-rabbit IgG conjugated with peroxidase 1:10 in 5 volumes PBS and 5 volumes normal human serum for 30 min at 37°C in a humidified atmosphere and washed twice with PBS for 5 min each. For localisation of the primary antibody 1 mg/ml of 3,3'- Diaminobenzidine tetra-hydrochloride solution was used. The sections were developed for 10 min at room temperature and then counterstained with Harris Hematoxylin. Control slides omitting the first antibody were used as negative controls in the immunohistochemical analysis of the p53 protein. The CM-1 antibody (36) was used with 15 specimens using the ABC technique as previously described (37). Two cell lines were used as control for p53 immunohistochemistry: the spontaneously immortalized rat 208F cells were used as negative controls for p53 expression and their transfected derivative RFV53HO6-3 cells, which carry the mutant mouse p53 gene carrying valine instead of alanine at amino acid 135, were used as positive controls. RFV53HO6-3 cells were derived after co-transfecting with the plasmid LTRp53cG-val containing the mutant p53 gene (38) and Homer 6 (34).

The immunostained sections for *ras* p21 or p53 were scored as (-/+) negative or equivocal; (+) moderate; (++) intense.

K-ras mutations.

DNA samples. DNA from 15 histological slides, fixed by formol and embedded in paraffin, was extracted by boiling in a lysis mixture.

Oligonucleotides primers and probes. The oligonucleotides were synthesized by the solid phase triester method. The primers were designed to introduce base substitution in the amplified fragments (39).

Polymerase chain reaction. In vitro enzymatic DNA amplification (PCR) was performed on an automated apparatus (DNA thermal Cycler from Perkin Elmer Cetus). We performed 35 cycles of amplification. Each cycle consisted of 3 steps: (i) denaturation of DNA at 94°C for 30 sec. (ii) annealing of the primers at 53°C for 30 sec. (iii) enzymatic extension at 72°C for 1 min.

Modified primers were designed to introduce a base substitution adjacent to the codon of interest in order to create an artificial restriction site with only one allelic form (wild type or mutated). We performed PCR with a modified primer creating a Msp I recognition site only if codon 12 was of the wild type. This approach allowed us to screen for point mutations at codon 12 of K-*ras* oncogene.

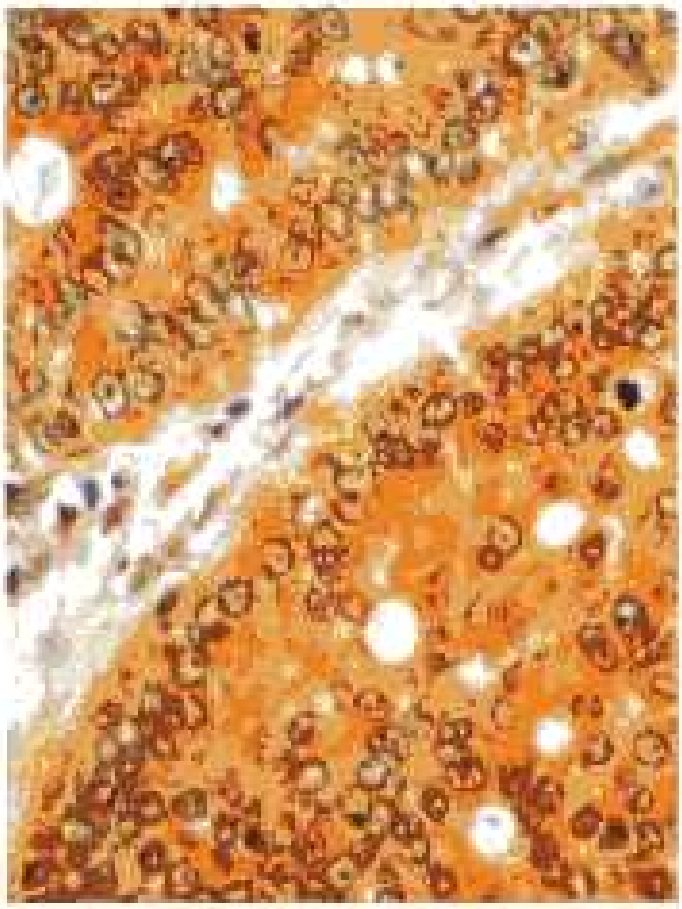
An aliquot of the PCR product was examined in a 2-3% Nu Sieve gel for the presence of the amplified fragment (99 bp). The PCR products were digested by Msp I enzyme which gave two fragments of 21 and 78 bp in all the 15 tumor DNA samples tested (all of them were wild type for codon 12 of the K-*ras* oncogene).

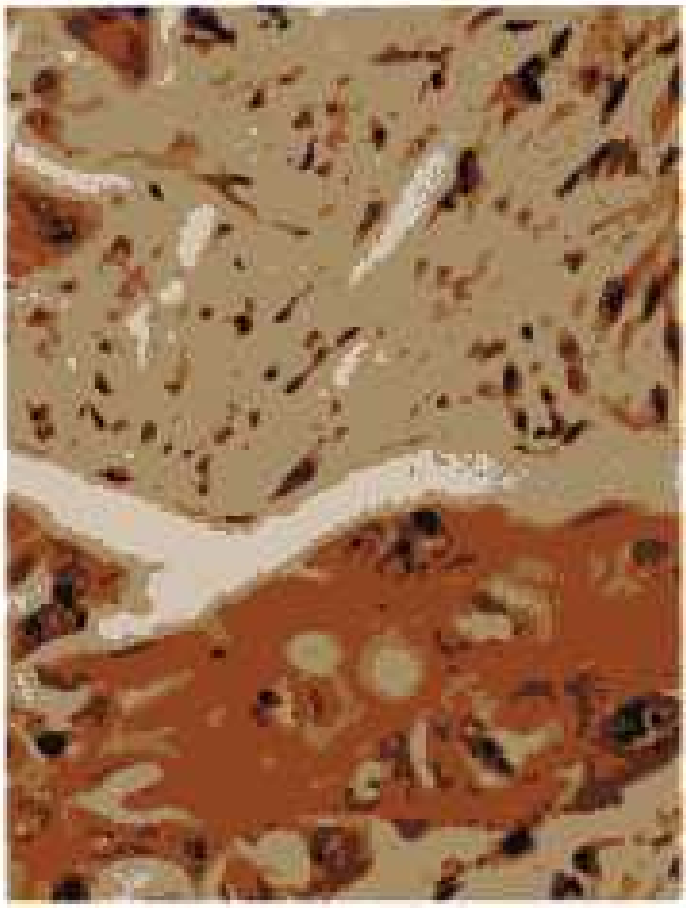
Statistical analysis. Quantitative data were analyzed by χ^2, Fisher's exact test or McNemar's χ^2, where appropriate. Survival curves were drawn up using the Kaplan-Meier product limit estimate (40). Differences between survival times were analyzed by the log rank method (41).

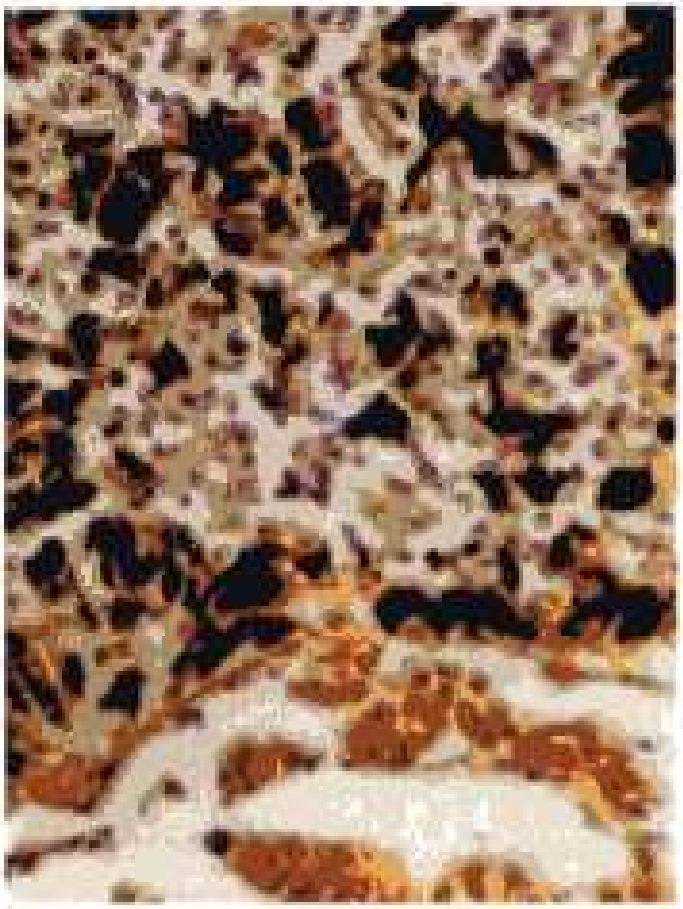
Results

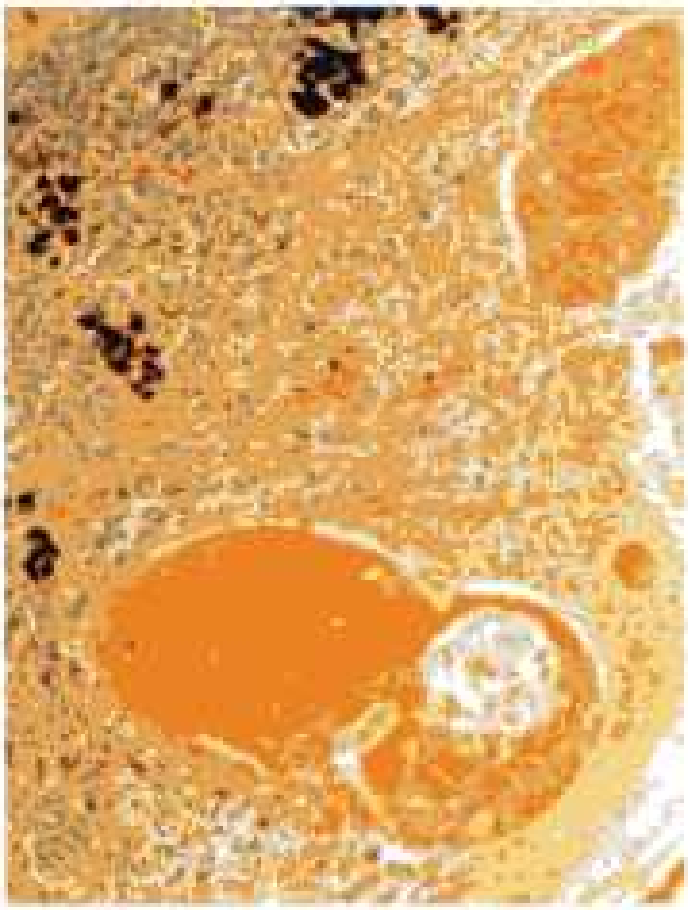
Forty-six NSCLC tumors were investigated for *ras* p21 and p53 expression. The *ras* p21 monoclonal antibody Y13-259 demonstrated good cytoplasmic staining in the positive scored tumor cells, and no staining in the normal cells (Fig. 1-4). The monoclonal antibody against p53, pAb 240 gave mainly diffuse staining in a large number of the positively stained tumor cells (Fig. 5-8). Similar staining patterns were found using the CM-1 antibody in 80% of cases. This result is consistent with the finding that CM-1 recognizes both wild type and mutant p53 protein.

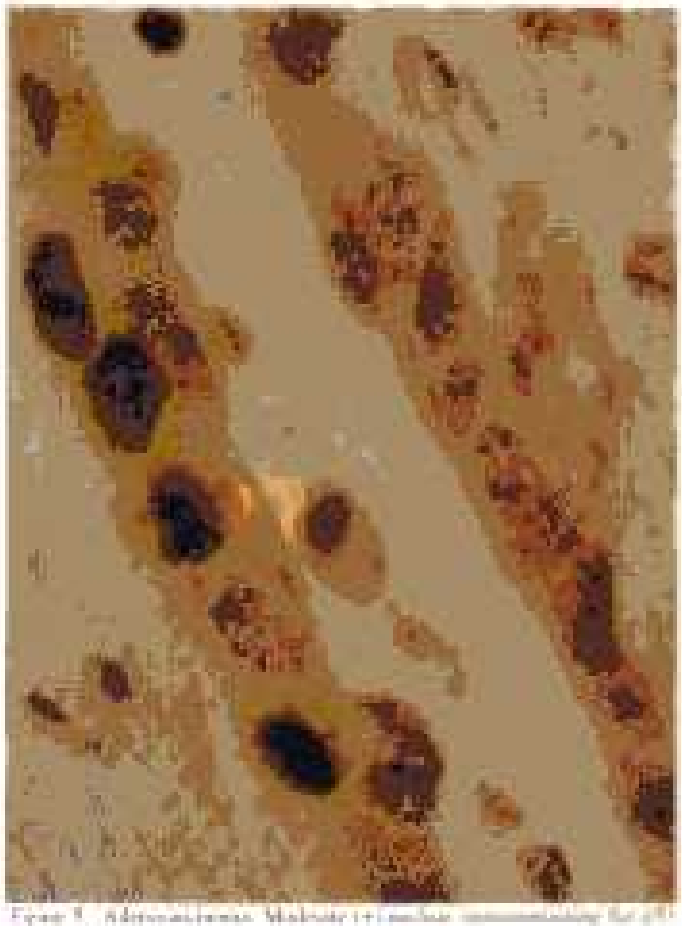
Ras p21 expression was elevated in 85% of the NSCLC tumors investigated. 90% of the adenocarcinomas and 81% of the squamous cell lung carcinomas had positive staining (Table I). Fifty-seven per cent of the NSCLC stage I; 94% of

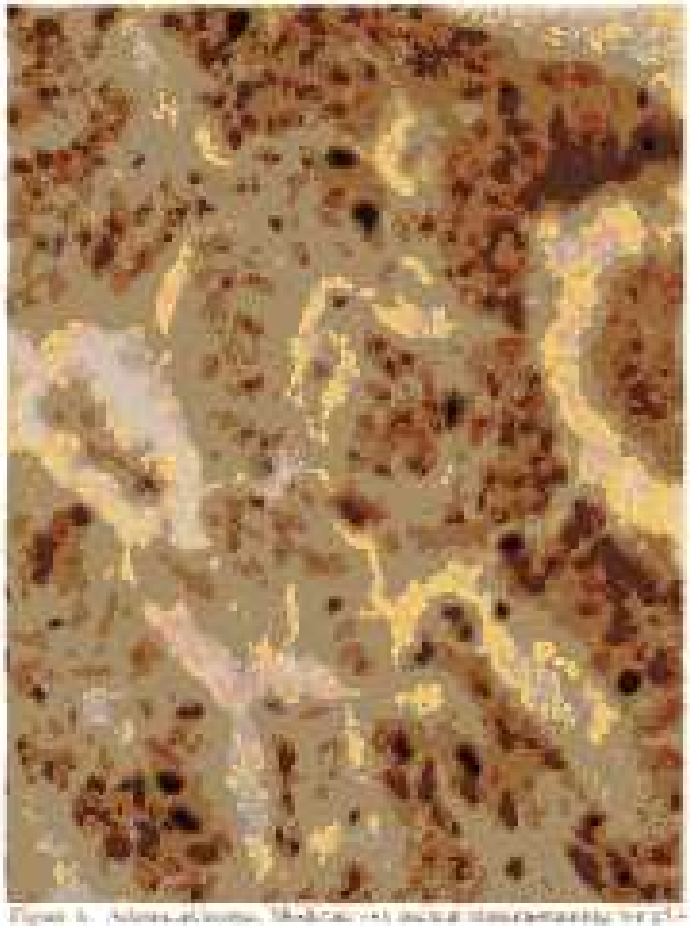


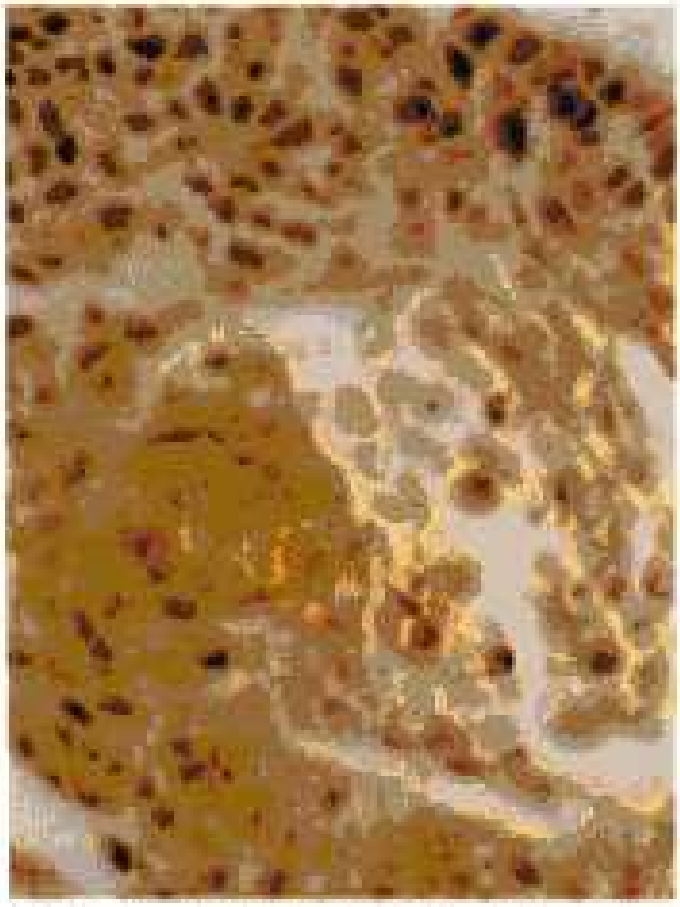












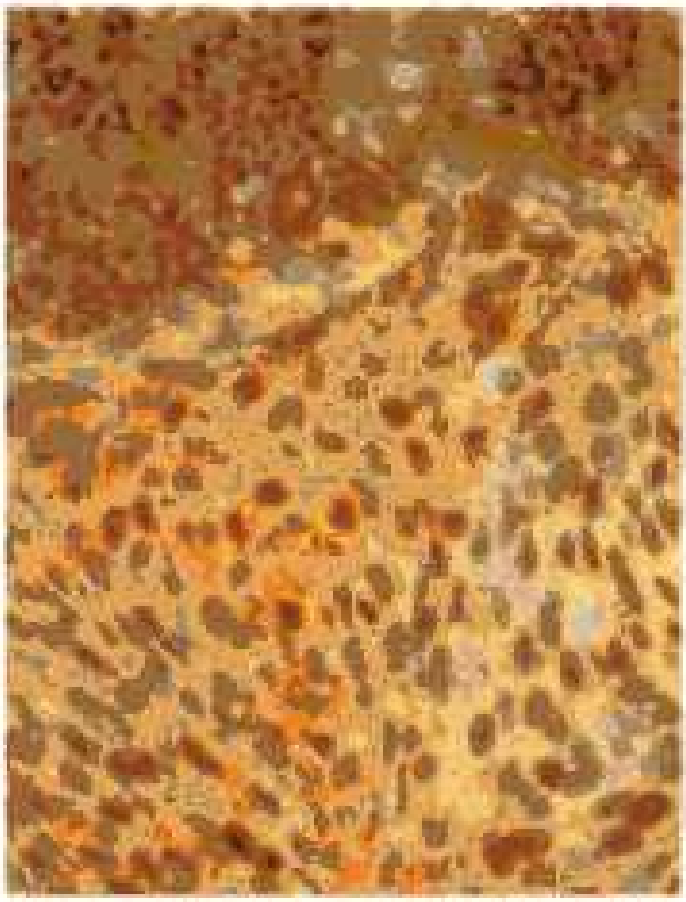


Table I. *Ras* and p53 expression in non-small cell lung carcinomas.

Tumor type	Total No	Intensity of staining/No of cases (%) *ras* p21 -/+	+	++	Intensity of staining/No of cases (%) p53 -/+	+	++
NSCLC [a]	46	7	17 (37)	22 (48)	25	16 (35)	5 (11)
Adenocarcinoma	20	2	9 (45)	9 (45)	11	6 (30)	3 (15)
Squamous cell carcinoma	26	5	8 (31)	13 (50)	14	10 (38)	2 (8)

[a] Non Small Cell Lung Carcinoma.

Table II. *Ras* p21 and p53 expression in tumor and lymph node metastasis of surgically treated NSCLC patients according to stage.

Stage	Total No	Tumor Intensity of staining/No of cases (%) *ras* p21 -	+	p53 -	+	Lymph nodes Intensity of staining/No of cases (%) *ras* p21 -	+	p53 -	+
I	14	6	8 (57)	12	2 (14)	14	0 (0)	14	0 (0)
II	18	1	17 (94)	10	8 (44)	1	17 (94)	15	3 (17)
IIIa	14	0	14 (100)	3	11 (79)	4	10 (71)	5	9 (64)

Fisher's exact tests.

Tumor data:
Stage I and stage (II+III)
ras p<0.05
p53 p<0.01

Stage (I+II) and stage III
ras p<0.05
p53 p<0.01

Lymph node data:
Stage I and Stage (II+III)
ras p<0.001
p53 p<0.01

Stage (I+II) and stage III
ras p>0.05
p53 p<0.001

stage II and 100% of stage IIIa stained *ras* p21 positive (Table II). 80% of stage I, 88% stage II and 100% of stage IIIa adenocarcinomas stained positive whereas 44% of stage I, 100% of stage II and 100% of stage IIIa squamous cell lung carcinomas stained *ras* p21 positive (Table III).

p53 expression was elevated in 46% of the NSCLC investigated. 45% of the adenocarcinomas and 46% of the squamous cell lung carcinomas stained positive with the pAb 240 monoclonal antibody (Table I). 14% of NSCLC stage I, 44% stage II and 79% stage IIIa tumors stained positive for p53 (Table II). 20% of the stage I, 50% of stage II and 57% of stage IIIa adenocarcinomas stained positive for p53 whereas 11% of stage I, 40% of stage II and 100% of stage IIIa squamous cell carcinomas stained positive for pAb 240 (Table III).

We also studied *ras* p21 and p53 expression in lymph nodes from surgically treated NSCLC patients. *Ras* p21 was over-expressed in 0% stage I; 94% stage II and 71% stage IIIa, while p53 was over-expressed in 0% stage I, 17% stage II and 64% stage IIIa in the lymph node specimens (Table II). Fifteen cases of benign lung lesions were also investigated for *ras* p21 and p53 expression; all were found to be negatively stained (Table IV).

Ras p21 and p53 staining in NSCLC were statistically analyzed with regard to staging of the tumor and lymph node specimens (Table II). Due to the small number of patients in certain categories, the staging data was analyzed by dividing them into two different groups; stage I and stage (II and IIIa), and stage (I+II) and stage IIIa. *Ras* p21 and p53 staining was found to be significantly different in

Table III. *Ras* p21 and p53 staining in tumor and lymph node metastasis of adenocarcinomas and squamous cell carcinomas of the lung according to tumor stage.

Stage	Adenocarcinomas Total No	Intensity of staining/No of cases (%) *ras* p21 -	*ras* p21 +	p53 -	p53 +	Squamous cell carcinomas Total No	Intensity of staining/No of cases (%) *ras* p21 -	*ras* p21 +	p53 -	p53 +
TUMOR										
I	5	1	4 (80)	4	1 (20)	9	5	4 (44)	8	1 (11)
II	8	1	7 (88)	4	4 (50)	10	0	10 (100)	6	4 (40)
IIIa	7	0	7 (100)	3	4 (57)	7	0	7 (100)	0	7 (100)
LYMPH NODE METASTASIS										
I	5	5	0 (0)	5	0 (0)	9	9	0 (0)	9	0 (0)
II	8	1	7 (88)	7	1 (13)	10	0	10 (100)	8	2 (20)
IIIa	7	2	5 (71)	5	2 (29)	7	2	5 (71)	0	7 (100)

Fisher's exact test: Stage I and stage (II and IIIa): *ras* ($p<0.05$) p53 ($p<0.05$)

Table IV. Immunohistochemical analysis of *ras* p21 and p53 protein in benign lung lesions.

Type of benign lesion	Total No.	Intensity of staining/No of cases *ras* p21 -	*ras* p21 +	Intensity of staining/No of cases p53 -	p53 +
Echinococcus	5	5	0	5	0
Pneumonia	4	4	0	4	0
Amartoma	3	3	0	3	0
Cyst	1	1	0	1	0
Necrosis	1	1	0	1	0
Granulomatous tissue	1	1	0	1	0

the subgroups stage (I+II) and stage IIIa for both tumor and lymph node tissue. However, *ras* p21 staining was only found to be significantly different in the tumor specimens. These data indicate that there is a relationship between increasing stages of the disease with both *ras* and p53 expression. When the data is further subdivided into adenocarcinomas and squamous cell carcinomas (Table III), it can be seen that a similar overall relationship exists between both *ras* p21 and p53 staining and stage of the disease.

Ras p21 and p53 staining was also analyzed in the NSCLC according to grade of differentiation (Table V). No statistically significant correlations were found. Also when subdivided into adenocarcinomas and squamous cell carcinomas no correlations were found between stage and grade of the NSCLC specimens analyzed in this study (Table VI). No correlations were found between *ras* p21 and p53 expression in this group of NSCLC patients (Table VII).

The patients' smoking history and the duration of smoking data was available for 45 or these NSCLC patients (Table VIII). All but two of these patients were either moderate or heavy smokers, the smoking duration fell into a range 10-60 years. No correlation was found between the NSCLC patients history of smoking (Table IX) or with duration (Table VIII).

Survival analysis. Stage of the disease in the NSCLC patients was found to correlate with a poor prognosis ($\chi^2=29.8$, df=2 $p<0.001$). No correlation was found for grade of differentiation with survival ($\chi^2=0.65$, df=2, $p>0.05$).

Ras p21 expression was found to be associated with survival ($p<0.1$) in both the tumor ($\chi^2=3.6$, $p<0.1$) (Fig. 9) and lymph node specimens ($\chi^2=3.1$, $p<0.1$) (Fig. 10). However, p53

Table V. *Ras* p21 and p53 expression in NSCLC according to grades of differentiation.

Grade	Total No.	Intensity of staining/No of cases			
		ras p21		p53	
		-	+	-	+
Low	13	3	10	9	4
Modeare	30	5	25	15	15
High	3	0	3	2	1

Fisher's exact test: *ras* p21 ($p>0.05$); p53 ($p>0.05$)

Discussion

We have examined 46 NSCLC primary tumor and nodal tissue specimens for *ras* p21 and p53 expression. Eighty-five per cent of these tumors expressed *ras* p21 with the Y13-259 antibody whereas 46% demonstrated elevated p53 mutant protein using pAb 240 monoclonal antibody. It is of particular note that elevated p53 expression correlated with survival in both the tumor ($p<0.05$) and also in lymph node metastasis specimens ($p<0.005$). This is the first publication that we know of which indicates a correlation between mutant p53 expression and a poor prognosis in NSCLC patients. The over-expression of *ras* p21 in these tumors was also found to be associated with a poor survival but was not statistically significant. However, Miyamoto *et al* (42) have demonstrated a correlation between *ras* p21 and survival using the *ras* rp-35 monoclonal antibody in a large survey of 112 NSCLC patients.

Table VI. *Ras* p21 and p53 staining in adenocarcinomas and squamous cell carcinomas of the lung according to grades of differentiation.

Grade	Adenocarcinomas					Squamous cell carcinomas				
	Total No	Intensity of staining/No of cases				Total No	Intensity of staining/No of cases			
		ras p21		p53			*ras* p21		p53	
		-	+	-	+		-	+	-	+
Low	6	0	6	4	2	7	3	4	5	2
Moderate	13	3	10	7	6	17	2	15	8	9
High	1	0	1	1	0	2	0	2	1	1

Table VII. Interrelationship between the expression of *ras* p21 and p53 in 46 NSCLC patients.

Intensity of staining/ No of cases		Intensity of staining/No of cases	
		ras p21	
		-	+
p53	-	5	20
	+	2	19

McNemar's χ^2 test; *ras* p21 and p53 staining results of the tumor data $p<0.001$

over-expression was found to correlate with a poor prognosis in the tumor specimens ($\chi^2=3.9$, $p<0.05$) (Fig. 11) and was found to be highly significant in lymph node tissues ($\chi^2=10.6$, $p<0.005$) (Fig. 12). No correlation was found between survival and smoking in these NSCLC patients.

Mutations in the K-ras gene. Mutations in K-*ras*, codon 12 has been assessed in a further group of 15 NSCLC patients, 6 adenocarcinomas, and 9 squamous cell carcinomas. None of these patients demonstrated a mutation in K-*ras* codon 12.

A number of clinico-pathological parameters including stage, grade of differentiation as well as the patient's smoking history were analyzed, in relation to the expression of *ras* p21 and p53 proteins.

In this study, stage of the disease was shown to correlate with a poor prognosis, as p53 and also *ras* p21, to some degree, correlated with survival, it is not surprising that the over-expression of these two genes correlated with the stage of the disease (Table II). This correlation was found in both the tumor and lymph node tissue data. On subdividing the NSCLC into squamous cell carcinomas and adeno-carcinomas this association was again found (Table III). No association was found for either *ras* p21 or p53 expression with the grade of differentiation of the NSCLC (Table V).

In this investigation we have demonstrated *ras* p21 over-expression in 81% of the squamous cell carcinomas compared to 90% of the adenocarcinomas. Spandidos *et al* (17) have previously reported significant differences between these two histological groups of NSCLC tumors. Similar findings were obtained from Rodenhuis *et al* (10) and Shiraishi *et al* (43) but not Kurzrock *et al* (44) and Koutselini *et al* (18) who found increased levels of *ras* p21

Table VIII. *Ras* p21, p53 clinicopathological parameters, smoking history and follow up on the 46 NSCLC patients analyzed in this study.

Pat. No.	Stage	Type	Grading	Sex	Smoking history		Follow up (months)	Fate
					Type	Duration		
1	I	AD	moderate	m	H	40	52	A
2	I	SQ	low	m	H	40	47	A
3	I	AD	moderate	m	H	35	52	A
4	I	SQ	moderate	m	M	25	39	A
5	I	SQ	low	m	M	30	38	A
6	I	SQ	moderate	m	H	30	26	A
7	I	AD	low	m	H	20	13	D
8	I	SQ	low	m	H	30	26	A
9	I	SQ	moderate	m	M	25	30	A
10	I	SQ	moderate	m	H	40	50	A
11	I	AD	moderate	m	M	50	23	D
12	I	AD	moderate	m	M	43	28	A
13	I	SQ	low	m	H	20	48	A
14	I	SQ	moderate	m	H	50	14	D
15	II	AD	moderate	m	H	50	26	D
16	II	SQ	moderate	m	H	45	10	D
17	II	SQ	moderate	m	M	45	28	A
18	II	AD	moderate	m	H	30	36	A
19	II	SQ	moderate	m	H	30	42	A
20	II	SQ	high	m	H	30	36	A
21	II	AD	moderate	m	ND	ND	23	D
22	II	SQ	high	m	H	50	35	A
23	II	AD	low	m	H	50	30	D
24	II	SQ	low	m	M	50	25	D
25	II	SQ	moderate	m	H	38	23	D
26	II	SQ	moderate	m	N	ND	36	A
27	II	SQ	low	m	H	50	48	A
28	II	AD	low	m	H	50	60	A
29	II	AD	high	m	M	50	38	D
30	II	SQ	moderate	m	H	30	30	A
31	II	AD	moderate	m	H	20	60	A
32	II	AD	moderate	m	M	60	39	D
33	IIIa	SQ	moderate	m	M	40	20	D
34	IIIa	SQ	moderate	m	H	40	6	D
35	IIIa	SQ	low	m	M	25	18	D
36	IIIa	AD	moderate	m	M	40	5	D
37	IIIa	AD	low	f	N	ND	24	D
38	IIIa	AD	moderate	m	M	10	26	A
39	IIIa	AD	moderate	m	H	20	4	D
40	IIIa	SQ	moderate	m	H	40	18	D
41	IIIa	AD	low	m	M	40	12	D
42	IIIa	AD	moderate	m	H	21	6	D
43	IIIa	AD	low	m	H	30	18	D
44	IIIa	SQ	moderate	m	H	25	11	D
45	IIIa	SQ	moderate	m	M	30	6	D
46	IIIa	SQ	moderate	m	M	40	17	D

Stage: New International Staging System for lung cancer (31). Type of carcinoma: AD = adenocarcinoma; SQ = Squamous cell lung carcinoma. Sex: m = male; f = female. Smoking type: non smoker (N); moderate smoker (M) (less than 20 per day); heavy smoker (H) (greater than 20 per day). Duration: duration of smoking habit in years. Fate: A = Alive; D = Dead; ND = No data.

oncoprotein in tumors with a squamous carcinoma histology using the same antibody.

Ras p21 expression was found in all stages of the disease but was particularly increased in stages II and IIIa in both the tumor and nodal tissue specimens. Rodenhuis *et al* (6) demonstrated that the adenocarcinomas with K-*ras* mutations tended to be in smaller tumors that had less often spread to the regional lymph nodes (6,10,12). The results of this investigation would indicate that *ras* over-expression was a late event in the progression of NSCLC patients.

In the present investigation p53 staining was found in 46% of NSCLC using the pAb 240 monoclonal antibody against the mutant p53 protein. This finding agrees with that of Hiyoshi *et al* (45) who used the p53 pAb 1801 antibody. They demonstrated p53 over-expression in 57% of the squamous cell carcinomas and in 43% of the adenocarcinomas. It is also

Table IX. Relationship between NSCLC patients smoking history and *ras* p21 and p53 expression.

	Intensity of staining /No of cases							
	ras p21				p53			
	Tumor		Node		Tumor		Node	
	-	+	-	+	-	+	-	+
Non smoker	0	2	1	1	0	2	1	1
Mod. smoker	3	13	8	8	8	8	12	4
Heavy smoker	4	23	10	17	17	10	20	4

of note that Hiyoshi *et al* (45) demonstrated that p53 staining correlated with regional node metastasis, distant metastasis and pathological stage in adenocarcinomas. Furthermore, in this present investigation p53 over-expression was found to significantly correlate with the stage of the disease, particularly in the lymph node specimens ($p<0.005$).

The role of smoking in lung cancer has been extensively reviewed (46). However, recent evidence also indicate a genetic link between the K-*ras*, p53 and a history of smoking. Rodenhuis *et al* (6) demonstrated that K-*ras* mutations were found in about one third of the adenocarcinomas of the lung investigated and that these mutations correlated with the patients smoking history. Furthermore, no other clinical correlations were noted with the K-*ras* mutations except the patient's smoking history. None of the non-smokers in their study had a mutation whereas 13 of 32 smokers did have a K-*ras* mutation. Similar results were found by Slebos *et al* (47). Kobayashi *et al* (7) have found a

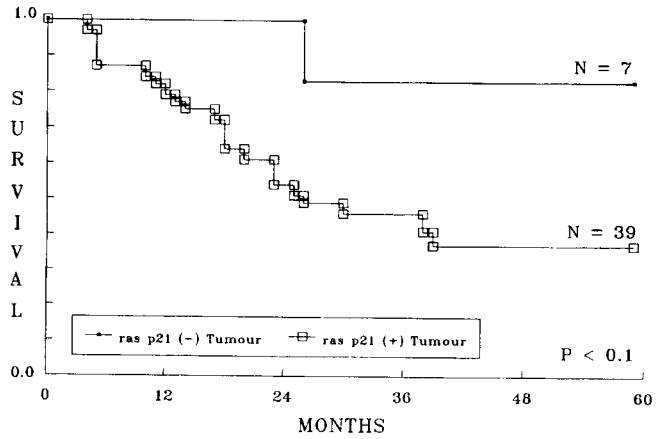


Figure 9. Survival curve of NSCLC tumor tissue for *ras* p21 expression, drawn up by Kaplan Meier (40) and log rank calculated by Peto (41).

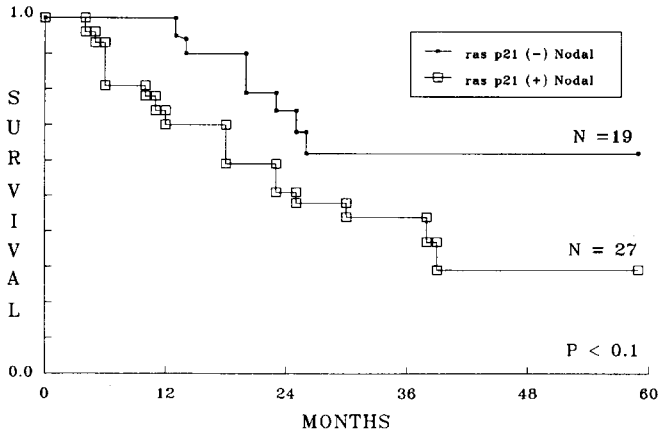


Figure 10. Survival curve of NSCLC nodal metastasis of surgically treated NSCLC patients for *ras* p21 expression, drawn up by Kaplan Meier (40) and log rank calculated by Peto (41).

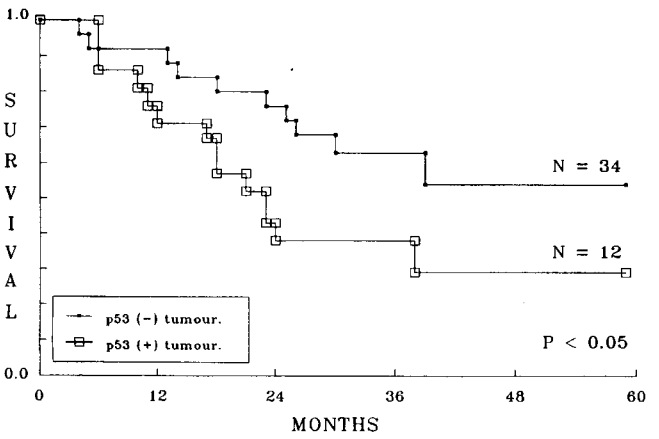


Figure 11. Survival curve of NSCLC tumor tissue for p53 expression, drawn up by Kaplan Meier (40) and log rank calculated by Peto (41).

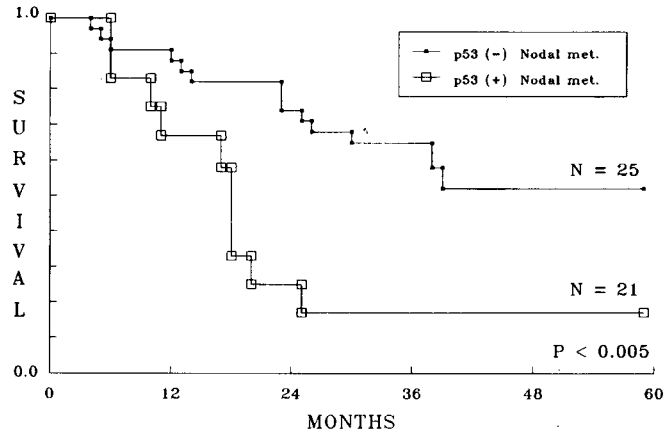


Figure 12. Survival curve of NSCLC nodal metastasis of surgically treated NSCLC patients for p53 expression, drawn up by Kaplan Meier (40) and log rank calculated by Peto (41).

higher incidence of K-*ras* mutations in NSCLC, than in SCLC and a correlation was found between these mutations and smoking habits in the non-goblet all types of these tumors. We also investigated the smoking history of the 45 NSCLC patients in this study, however, as only two patients were non-smokers it is not surprising no correlations were forthcoming.

The evidence associating p53 mutations and a history of smoking has become more compelling. Chiba *et al* (8) demonstrated that 56% of p53 mutations in the lung tumors were G to T transversions unlike many other tumors. It is believed that the type of mutation is usually associated with a specific mutagen, for example benzo (a) pyrene may cause G to T transversions in certain circumstances (48) whereas different mutagens can cause G to A transversions. This was the first molecular evidence which suggested that lung cancer is caused by a specific mutagen and may indicate that a particular carcinogen in the smoke causes lung cancer. Although Chiba *et al* (8) did not demonstrate a correlation between p53 mutations and a history of smoking, this data has been recently provided by a Japanese group (9) which clearly found a correlation between a life-time cigarette consumption and p53 mutations in lung cancer patients. It is also of particular interest that Field *et al* (49,50) have found a correlation of the head and neck cancer and a history of heavy smoking and drinking.

When these results are considered together, they suggest that the p53 gene may play a role in the early development of lung cancer. Since life-time cigarette consumption correlated with p53 mutations (9) and nodal metastasis, stage and poor prognosis also correlates with p53 overexpression (our data), it may be proposed that the aberrant expression of this gene has also a role in the late events in lung neoplasias.

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