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Prognostic significance of K-*ras*, p53, bcl-2, PCNA, CD34 in radically resected non-small cell lung cancers

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Abstract

The aim of this study was to investigate the prognostic significance of a panel of biological parameters in patients with radically resected non-small cell lung cancers (NSCLC). 269 cases with pathological stage I-IIIA NSCLC were retrospectively analysed. Immunohistochemistry was performed to detect protein expression of p53, bcl-2, proliferating cell nuclear antigen (PCNA) and CD34. Polymerase chain reaction (PCR)/direct nucleotide sequencing method was used to detect mutations in K-*ras* (codons 12, 13, 61, exons 1-2). The Kaplan–Meier estimates of survival were calculated for clinical and biological variables using the Cox model for multivariate analysis. Histological subtype and the pathologic tumour extension (pT) were the most powerful clinical–pathological prognostic factors for survival ($P=0.030$ and $P=0.031$, respectively), whereas among the biological parameters, p53 overexpression ($P=0.032$) and K-*ras* mutation ($P=0.078$) had a negative prognostic role, as demonstrated by multivariate analysis. Conversely, bcl-2, PCNA and CD34 expression were not correlated with survival. Statistically significant associations between p53 expression and the squamous cell carcinoma (SCC) subtype, bcl-2 expression and SCC subtype, K-*ras* mutation and p53 negative expression, p53 and bcl-2, bcl-2 and PCNA overexpression were observed. In conclusion, some biological characteristics such as the K-*ras* and p53 status may provide useful prognostic information in resected NSCLC patients, in addition to the classical clinico-pathological parameters. However, further studies are needed to clarify the value of adopting biological prognostic factor into clinical practice.

Keywords: Prognostic factors; K-*ras*; p53; bcl-2; PCNA; CD34; Biologic markers; Lung cancer

1. Introduction

Lung cancer is still the leading cause of cancer-related death worldwide. In 2002, it is expected to contribute to 31% of all cancer deaths in men and 25% in women [1]. Non-small cell lung cancer (NSCLC) represents the most frequent histological subtype of all lung tumour cases. Clinical outcome in these patients mainly depends on the disease stage at the time of diagnosis. Following radical resection, a 5-year survival probability up to 70% can be achieved in patients with node-negative disease. Nodal involvement correlates with a significantly poorer prognosis. Cure rates with surgery alone for stage II (N1) or resectable stage IIIA (N2) disease range from 20 to 50%. The identification of prognostic and predictive factors may provide hints for the selection of resectable patients who are at a higher risk for recurrent disease (prognostic factors), or who are more likely to benefit from adjuvant treatment (predictive factors).

In the past, many studies have identified a number of negative clinical prognostic factors including weight loss, poor performance status, presence of systemic symptoms, gender and age [2]. Other studies have

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reported the prognostic impact of a number of pathological characteristics such as tumour-node-metastasis (TNM) stage, histological subtype (squamous versus non-squamous), degree of differentiation and vascular invasion [3,4]. Unfortunately, knowledge regarding these negative clinical prognostic factors has not improved the efficacy of the treatment in NSCLC. Most of the expectations in this field rely on a better understanding of lung cancer biology and on the development of therapeutic strategies based on new drugs against novel molecular targets.

Recently, many genetic alterations which are implicated in the pathogenesis of cancer have been identified. Such alterations, including overexpression of certain oncogenes or inactivation of tumour suppressor genes, have been found in significant numbers of patients with lung cancer and are suitable candidates for prognostic or predictive factors.

Tumour growth results from an imbalance between proliferation (cell division) and cell loss (necrosis or apoptosis). Tumours grow both by increasing cell proliferation and/or by decreasing the rate of apoptotic cell death. Our research has focused on a panel of five biological indicators associated with apoptosis, cell proliferation and neoangiogenesis detected by immunochemistry and gene sequencing.

p53 gene mutations and abnormal expression of the bcl-2 gene product have been found in a variety of human tumours including NSCLC. The *p53* tumour-suppressor gene (altered in 40–74% of NSCLC [5], and *bcl-2* (abnormally expressed in 25–35% of squamous cell carcinomas and in approximately 10% of adenocarcinomas) [2] are involved in the regulation of apoptosis. p53 interferes with the expression of a number of proapoptotic genes at the transcriptional level; p53 induces bax and inhibits the expression of bcl-2, shifting the balance towards cell death. The bcl-2 gene product has been demonstrated to inhibit apoptosis, thereby prolonging cell survival. Overexpression of bcl-2 can inhibit p53-mediated G_1 arrest [5].

Mutations of *ras* occur in approximately 30% of all human cancers including NSCLC, particularly adenocarcinomas [6]. Many studies have suggested that the majority of these mutations are present in codon 12 of K-ras and that G to T transversions were the most common [7]. Mutated *ras* genes produce mutated proteins that remain in an active state, thereby relaying uncontrolled proliferative signals. Ras undergoes several posttranslational modifications that facilitate its localisation at the inner surface of the plasma membrane.

Recent studies have shown that tumour angiogenesis is an important prognostic factor in different types of cancer, including NSCLC [8,9]. Increase of microvascular density (MVD) is a predictor of poor survival [8]. The MVD has been measured with an anti-CD34 monoclonal antibody which is specific for endothelial cells and has been used to measure angiogenesis in tumour samples.

Tumour proliferative activity can be assessed using a monoclonal antibody which recognises a proliferating cell nuclear antigen (PCNA). Synthesis of PCNA correlates with the proliferation of cells in many human tumours, including NSCLC [10]. PCNA levels increase in late G1 phase and peak in the S phase of the cell cycle and the antigen is not detectable in quiescent cells.

We aimed at retrospectively assessing the prognostic relevance of p53, bcl-2, K-ras, CD34 and PCNA in addition to classical clinical–pathological prognostic factors in resected NSCLC samples.

2. Patients and methods

2.1. Patients' features

Patients with pathological stage I-IIIA NSCLC, submitted to radical resection in the Divisions of Thoracic Surgery of University Hospital and San Martino General Hospital of Genoa, were suitable, after pathological review, for this study. Sections were obtained from formalin-fixed, paraffin-embedded tissue blocks. Cases with inadequate or insufficient pathological material were excluded. Eligibility criteria included pathological stage I-IIIA, complete resection of the tumour, lobectomy (including sleeve lobectomy and bilobectomy), pneumonectomy and other (anatomical segmentectomy or wedge resection) along with *en bloc* lymphoadenectomy of at least two different mediastinal lymph node stations.

2.2. Pathological criteria for specimen selection and classification

Routinely formalin-fixed, paraffin-embedded tissue blocks from 269 patients, retrieved from the pathology archives of the Anatomical Pathology Divisions of the University Hospital and San Martino General Hospital of Genoa, were used in this study. The diagnosis of NSCLC was made by conventional haematoxylin and eosin staining. Tumour classification was performed according to the World Health Organization (WHO) [11] and to the Armed Forces Institute of Pathology (AFIP) criteria [12]. Tumour stage was determined according to the international TNM staging system (former version Mountain 1986) [13]. A single block from formalin-fixed and paraffin-embedded tissue, representative of each tumour specimen, was selected. Ten 4-μm sections were cut for immunohistochemical evaluation and two 15-μm sections were used for molecular diagnosis.

2.3. K-ras mutational analysis: DNA extraction, polymerase chain reaction, direct sequencing

2.3.1. DNA preparation

Two sections (15 μm) of formalin-fixed, paraffin-embedded tumour tissue sections were dissected following closely the distribution of tumour tissue in order to ensure that 90% of the section contained tumour tissue.

Such sections were treated with xylene and incubated overnight at 55 °C with 1 ml of extraction buffer (50 ml of the solution is composed of 1 ml of NaCl 5M, 0.5 ml of Tris/HCl 1M, 0.025 ml of ethylene diamine tetra acetic acid (EDTA) 0.5M, 1.25 ml of sodium dodecyl sulphate (SDS) 20%, 28.875 ml of ammonium acetate 7.5 M), and 30 μl of Proteinase K (10 mg/ml in ddH_2O). The DNA extraction was terminated with a phenol/chloroform step, followed by an ethanol precipitation. DNA pellets were suspended in 100 μl of ddH_2O. A qualitative (on a 0.8% agarose gel) and a quantitative check (spectrophotometric reading) of the DNA samples were performed.

2.3.2. PCR amplification

DNA extracted from tissue sections was a suitable template for the amplification of short target DNA fragments (from 80 to a maximum of 270 bp) The sequences of the specific primers used for the K-*ras* gene amplification are:

K-*ras* ex 1 Forward	5′-GACTGAATATAAACTTGT GGTAG3′
K-*ras* ex 1 Reverse	5′-CTATTGTTGGATCATAT TCG-3′
K-*ras* ex 2 Forward	5′-CCTACAGGAAGCAAGTA GTAAT-3′
K-*ras* ex 2 Reverse	5′-CACAAAGAAAGCCCTCC CCA-3′

Polymerase chain reaction (PCR) were conducted in a total volume of 50 μl, using the followed reagents: 5 μl 10× PCR buffer, deoxynucleotide triphosphates (dNTPs) 5 μl 10×, 5 μl dimethylsulphoxide (DMSO), 5 μl ($MgCl_2$ 25 mM), 12.5 pM primer forward, 12.5 pM primer reverse, 100 ng DNA, up to 50 μl with ddH_2O.

The PCR cycle profile was:

For K-*ras1* and K-*ras2*:

35 cycles: 94 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s.

The PCR reactions were analysed by agarose gel electrophoresis (2%), purified and used in two sequencing reactions per exon (a forward and a reverse sequencing reaction), using a Big Dye Terminator method and the reactions were loaded into an ABI Prism 377 Sequencer.

2.3.3. Data analysis

The raw sequencing data were analysed using two different software programs: one for the alignment with the consensus sequence of the normal gene, MT navigator (AB: Applied Biosystems 850 Lincoln Drive, Foster City, CA 94404, USA) and MatchTool (AB) for the identification of mutations.

2.4. p53, bcl-2, PCNA, CD34 expression analysis: immunohistochemistry

Immunohistochemical analysis of formalin-fixed, paraffin-embedded tissue was performed using the avidin-biotin complex immunoperoxidase method. Slides were deparaffinised in xylene, hydrated in graded alcohol and endogenous peroxidase activity was blocked by 30 min treatment with 3% hydrogen peroxide in absolute methanol at room temperature. Sections were then incubated in phosphate-buffered saline (PBS pH 7.4) and treated with the primary monoclonal antibodies specific for p53 (DO-7, Dako A/S Produktionsvej 42 DK 2600 Glostrup), bcl-2 (124 1:20, Dako A/S Produktionsvej 42 DK 2600 Glostrup), PCNA (PC-10 1:200, BioGenex 4600 Norris Canyon Road, San Ramon, CA 94583, USA), CD-34 (QB-END10 1:200, Ylem V. Gramsci 56 00197, Rome, Italy). Section were rinsed in PBS and incubated for 30 min with biotinylated secondary antibodies. Streptavidin-conjugated peroxidase (Strept ABComplex, Dako) was applied and, finally, sections were rinsed with PBS, developed with diaminobenzidine tetrahydrochloride substrate (DAB, Cromogen) for 3 min, and counterstained with haematoxylin.

The immunohistochemical evaluation was assessed by two observers on all sections. The immunostaining for PCNA expression was classified in three categories: 'negative' (including negative samples and samples with $<10\%$ positive cells), 'intermediate' (positive cells from 10 and 50%) and 'intense' (positive cells $>50\%$). In cases of p53 and bcl-2 stains, the staining intensity was considered as a principal factor and, for the protein expression, the score 'negative' was attributed to samples that were negative or weakly positive (+), the score 'intermediate' to samples with moderate staining intensity (++) and with $<50\%$ positive cells, the score 'intense' to samples with strong staining intensity (+++) or to tumours with moderate staining intensity (++) expressed in more than 50% of the neoplastic cells. MVD, evaluated by CD34 expression, was determined in the three areas of maximal vascularisation. Counting was performed at 250× magnification and CD34 expression was classified into three groups: <16, from 16 to <30 and $\geqslant 30$ microvessels/area. Damaged samples were considered 'not evaluable'.

2.5. Statistical analysis

The clinical variables considered were the following: gender, age, histological type, pathological TNM, tumour site, grading and type of surgery. Overall survival (OS) curves were calculated from the date of surgery, using death from any cause as the endpoint. Kaplan–Meier estimates were calculated for each clinical variable and biological marker [14] and were compared using the log-rank test [15]. Multivariate assessment of OS was performed by the Cox's proportional hazard model. Clinical–pathological characteristics with a univariate P value $\leqslant 0.20$ and all biological factors were initially included in the multivariate model. Variables not significantly associated with OS were removed from the model by means of a step-down procedure based on the likelihood ratio test. The significance level for removing a variable from the model was set at 0.10. Correlations between biological alterations and clinico/pathological features were assessed using the Chi-square or Fisher's Exact probability tests. In addition to a complete database, data were also analysed independently considering only the patients with stage I, squamous cell carcinoma (SCC) histological subtype and non-SCC histological subtype (data not shown).

3. Results

3.1. Patients and tumour characteristics

Between January 1990 and December 1996, 269 patients with resected NSCLC were selected. Formalin-fixed, paraffin-embedded tissue blocks were processed for K-*ras*, p53, bcl-2, PCNA and CD34. Patient and tumour characteristics are listed in Table 1. In our series, the majority of patients were male (90%) and had SCC (56%). Mean age was 64 years (range 38–81 years). Approximately half of the patients (52%) had stage I disease.

Table 1 summarises results concerning the frequency of the various biological factors examined. In particular, K-*ras* was wild-type in 76% of patients, whereas 18% had mutations; at codon 12 (73%), codon 13 (6%) or codon 61 (21%). At codon 12, the G to T transversion (GGT, Gly→GTT, Val) was the most common genetic alteration and the mutation was frequently heterozygous. As for p53 expression, 55% of tumour samples were negative, 9% were moderately positive and 26% were strongly positive. The immunostaining pattern was mainly nuclear. Bcl-2 protein expression was negative in 63% of samples, moderately positive in 9% and strongly positive in 21%. The PCNA immunoreactivity was negative in 51% of tumours, moderately positive in 23% and strongly positive in 19%. Microvessel number, as measured by CD34 immunostaining, was <16 in 22%, from 16 to <30 in 53% and $\geqslant 30$ in 16% of the samples.

Table 1
Distribution of patients by clinical–pathological and biological characteristics

Characteristic	*N* (%)
Total	269 (100)
Gender	
Males	242 (90)
Females	27 (10)
Age (years)	
<70	189 (70)
$\geqslant 70$	80 (30)
Histology	
Squamous (SCC)	151 (56)
Non-squamous (N-SCC)	118 (44)
Tumour site	
Right lung	156 (58)
Left lung	113 (42)
Stage	
I	141 (52)
II	46 (17)
IIIA	82 (30)
Grading	
1	33 (12)
2	160 (59)
3	76 (28)
Type of surgery	
Pneumonectomy	48 (18)
Lobectomy	214 (80)
Other (segment or wedge)	7 (3)
K-*ras* (265 tested)	
Wild-type	202 (76)
Mutant	47 (18)
Not evaluable	16 (6)
p53 (234 tested)	
Negative	128 (55)
+ +	22 (9)
+ + +	60 (26)
Not evaluable	24 (10)
bcl-2 (230 tested)	
Negative	144 (63)
+ +	20 (9)
+ + +	49 (21)
Not evaluable	17 (7)
PCNA (239 tested)	
Negative	121 (51)
+ +	54 (23)
+ + +	45 (19)
Not evaluable	19 (8)
CD34 (222 tested)	
MVD <16	48 (22)
$16 \leqslant$ MVD <30	117 (53)
MVD $\geqslant 30$	35 (16)
Not evaluable	22 (10)

Not evaluable: inadequate sample; + +/+ + +: see description in the text; MVD: microvascular density; PCNA, proliferating cell nuclear antigen.

3.2. Survival according to the main clinical/pathological prognostic factors

The overall 5-year survival rate for the whole series was 54.8%, (53.0% for males and 71.0% for females). Only 4 patients out of 269 (1.5%) died within 30 days from surgery and were not censored in the survival analysis.

The histological subtype and pathological stage were independent prognostic factors for survival. In particular, the 5-year survival rate for SCC was 60.0%, as opposed to 49.0% for non-squamous cell carcinomas (N-SCC) ($P=0.030$). The 5-year survival rate was 63.0% for patients with stage I disease, compared with 53.5% for patients with stage II and 42.2% for patients with stage IIIA disease ($P=0.031$). Tumour site, as well as grading and type of surgery, had no statistically significant impact on survival (Table 2). Table 2 lists the results of the univariate analysis for the prognostic significance of each factor analysed in relation to survival,

Table 2
Five-year survival probabilities estimated with the Kaplan–Meier method according to clinical–pathological and biological characteristics (univariate analysis)

Characteristic	*N*	5-year OS (%)	HR (95% CI)	*P* value
Total	269	54.8	–	–
Gender				
Males	242	53.0	1 (Ref.)	0.091
Females	27	71.0	0.54 (0.26–1.11)	
Age (yrs)				
<70	189	56.9	1 (Ref.)	0.485
$\geqslant 70$	80	50.4	1.15 (0.78–1.69)	
Histology				
Squamous (SCC)	151	60.0	1 (Ref.)	0.030
Non-squamous (N-SCC)	118	49.0	1.48 (1.03-2.11)	
Tumour site				
Right lung	156	51.2	1 (Ref.)	0.307
Left lung	113	60.5	0.83 (0.57–1.19)	
Stage				
I	141	63.0	1 (Ref.)	0.031
II	46	53.5	1.33 (0.82–2.16)	
IIIA	82	42.2	1.70 (1.14–2.53)	
Grading				
1	33	50.5	1 (Ref.)	0.422
2	160	59.4	0.77 (0.46–1.30)	
3	76	48.9	0.97 (0.56–1.69)	
Type of surgery				
Pneumonectomy	48	44.0	1 (Ref.)	0.139
Lobectomy/Other	214	57.5	0.72 (0.46–1.12)	
K-*ras* (265 tested)				
Wild-type	202	57.9	1 (Ref.)	0.078
Mutant	47	41.5	1.46 (0.96–2.22)	
p53 (234 tested)				
Negative	128	54.4	1 (Ref.)	0.360
$++/+++$	82	51.1	1.11 (0.89–1.38)	
bcl-2 (230 tested)				
Negative	144	61.8	1 (Ref.)	0.211
$++/+++$	69	49.5	1.16 (0.92–1.47)	
PCNA (239 tested)				
Negative	121	54.3	1 (Ref.)	0.312
$++$	99	58.0	0.87 (0.67–1.13)	
CD34 (222 tested)				
MVD <16	48	58.3	1 (Ref.)	0.974
MVD $\geqslant 16$	152	57.0	0.99 (0.71–1.39)	

OS, overall survival; HR, hazard ratio; *P*, significance level for log-rank test; $++/+++$, see description in the text; MVD, microvascular density; 95% CI, 95% Confidence Interval; Ref., reference group; PCNA, proliferating cell nuclear antigen.

expressed as risk ratios, 95% Confidence Intervals (CI) and *P* values.

3.3. Survival according to biological prognostic factors

Among the examined biological factors, K-*ras* mutation seems to be an unfavourable prognostic factor, with 57.9% 5-year survival for patients expressing the wild-type gene and 41.5% for patients carrying the mutated gene, although the statistical comparison was marginally significant ($P = 0.078$) (Table 2 and Fig. 1).

In the multivariate analysis, only histology ($P = 0.003$), clinical stage ($P = 0.073$), K-*ras* ($P = 0.078$) and p53 expression ($P = 0.032$) were retained in the final model (Table 3).

In addition, data analysis which considered only the group of patients with histological subtype SCC and N-SCC, demonstrated that the negative prognostic effect of p53 alteration was highly significant in patients

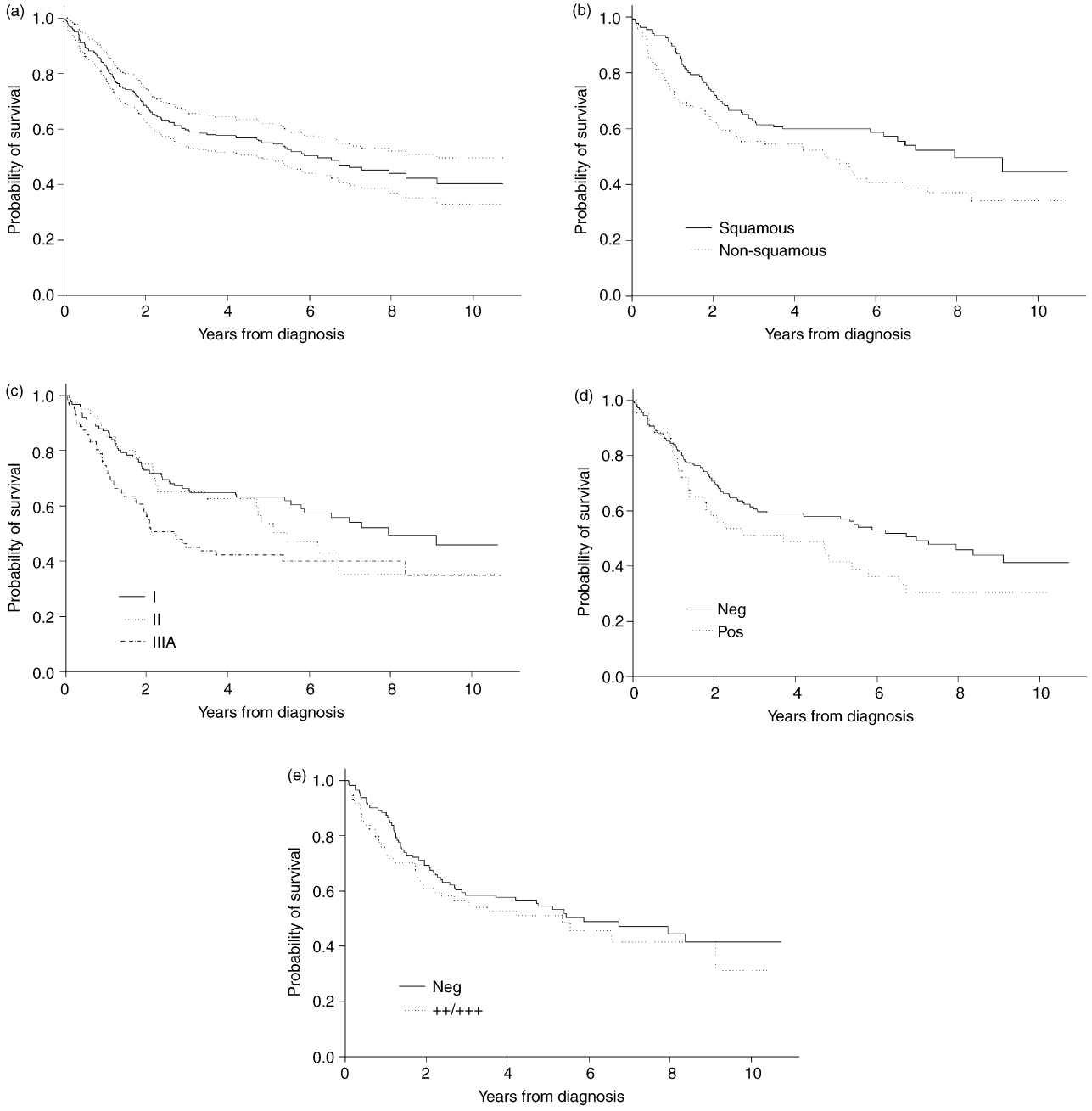

Fig. 1. Survival curves: (a) overall survival (including 95% Confidence Intervals (CIs), (b) probability of overall survival by histological subtype, (c) overall survival by clinical stage, (d) overall survival by K-*ras*, and (e) overall survival by p53. Neg, wild-type; Pos, mutated.

Table 3
Results of the multivariate analysis by means of the Cox's proportional hazard model

Characteristic	HR (95% CI)	*P* value
Histology		
Squamous (SCC)	1 (Ref.)	0.003
Non-squamous (N-SCC)	2.13 (1.29–3.51)	
Stage		
I/II	1 (Ref.)	0.073
IIIA	1.54 (0.96–2.47)	
K-*ras*		
Wild-type	1 (Ref.)	0.078
Mutant	1.66 (0.94–2.93)	
p53		
Negative	1 (Ref.)	0.032
+ +/+ + +	1.74 (1.05–2.89)	

HR, hazard ratio; *P*, significance level for Wald test; + +/+ + +, see description in the text.

with adenocarcinoma, but not in patients with SCC ($P = 0.001$ versus $P = 0.8$).

3.4. Associations

The percentage of p53 and bcl-2 expression (+ +/+ + +) was strongly associated with the histological subtype, with a higher expression in SCC than in N-SCC ($P < 0.05$). K-*ras* mutation was associated with type of surgery (pneumonectomy) ($P < 0.05$). Microvessel counting $\geqslant 16$ was correlated with an age $\geqslant 70$ years ($P = 0.07$).

Among the different biological features, p53 and bcl-2 expression (+ +/+ + +) were positively correlated (odds ratio (OR) = 10.7, $P < 0.001$), as well as bcl-2 and PCNA expression (OR = 1.86, $P < 0.038$). In contrast, an inverse correlation was found between K-*ras* mutations and p53 expression (association between mutated K-*ras* and p53 negative expression, OR = 0.47, $P = 0.057$) (data not shown).

4. Discussion

In recent years, the rapidly growing knowledge about the biology of lung cancer has led to the identification of a number of new molecular prognostic factors that may help clinicians to better assess survival probabilities and to optimise therapeutic strategies so that they are individually tailored using novel targeted therapies.

Among the clinical parameters evaluated in our study, histological subtype and pathological TNM staging had a statistically significant relevance for prognosis. As seen in the majority of other published studies, patients with SCC and earlier stage disease had a more favourable outcome [3,2]. Most studies have evaluated only one or a few biological factors, and the correlation among the various biological markers has not been properly assessed. Of the five biological factors examined in our study, only p53 protein overexpression and K-*ras* mutation had an independent unfavourable prognostic significance.

Several other studies assessing the relevance of these molecular markers for lung cancer prognosis have been reported with conflicting results. For p53 protein accumulation, many authors have shown a negative role for survival of NSCLC patients [16–18], while others did not find any correlation with prognosis [16,19–22], or even indicated that p53 was a favourable prognostic factor [23,24]. A recent meta-analysis, including 43 published papers [16], showed, similar to our results, that the incidence of p53 overexpression and mutations was significantly lower in adenocarcinoma than in SCC. In addition, the negative prognostic effect of p53 alteration was highly significant in patients with adenocarcinoma, but not in patients with SCC, as also noted in our analysis. We observed, when all histological subtypes were considered, that p53 overexpression was an unfavourable prognostic factor in multivariate, but not in univariate analysis. A possible explanation for this discrepancy is an imbalance in distribution resulting from the association of p53 overexpression with SCC histological subtype and with K-*ras* wild-type conditions, which confer a better prognosis. The correlation we found between p53 and the squamous subtype was also reported by some authors [16,25]. In addition, other studies, like ours, demonstrated a correlation between p53 and bcl-2 overexpression ($p53^+/bcl\text{-}2^+$ and $p53^-/bcl\text{-}2^-$) [12,26]. In contrast, Fontanini and Silvestrini reported an inverse correlation [27,28].

In our study, K-*ras* mutations were also associated with a poor prognosis. Univariate and multivariate analyses indicated better survival for patients with wild-type K-*ras* (57.9% versus 41.5% $P = 0.078$), as has also been reported in several studies [29–35], whereas others [36–39] have ruled out its correlation with prognosis. Contrary to several studies, where K-*ras* mutations have been found almost exclusively in patients with adenocarcinoma [6,29,30], we found mutations in both adenocarcinoma and SCC patients. In addition, in our study most point mutations in the K-*ras* gene consisted of G→T transversions in codon 12 of exon 1, as reported by others [32,33] but in contrast with findings of Cho and colleagues who reported G→A transitions as the most frequent mutation [40]. Keohavong and colleagues [37] suggested that the type of K-*ras* mutation at codon 12 is relevant for the prognosis of lung cancer patients. A substitution of the wild-type glycine at codon 12 with valine or arginine indicates a strong trend towards a poorer prognosis, while a substitution with aspartate appears to correlate with a better outcome.

Contrary to other studies, where K-*ras* mutations only looked for at codon 12, we also performed a K-*ras* of codons 13 and 61, and found mutations in these codons as well. This might partially explain our differing results for SCC. Interestingly enough, we found statistically significant associations between K-*ras* mutations and the type of surgery (pneumonectomy) and between K-*ras* mutations and p53 negative expression.

The prognostic role of bcl-2 in NSCLC is controversial, being found to be a favourable prognostic factor in some studies [27,41,42], but associated with unfavourable prognosis in others [16,43], or with no prognostic impact in some others [19], such as in our study. Furthermore, we found that bcl-2 strictly associates with p53 expression and with the squamous subtype ($P<0.05$), as has also been reported by others [44,45].

Proliferating cell nuclear antigen (PCNA) expression was not correlated with prognosis in our investigation, in accordance with results of other authors [45,46]. However, other studies have suggested that PCNA represents an unfavourable prognostic factor [10,25,47–50]. The angiogenic marker, CD34 (indicated by Vermeulen [51], together with CD31, as a better angiogenic marker than Factor VIII) was not correlated with prognosis in our study. Our results are in accordance with those of Pastorino and Decaussin [19,52], but contrast with those of Fontanini [8], who indicated CD34 to be an unfavourable prognostic factor.

In conclusion, our study indicates a possible added value, compared with standard clinical–pathological prognostic factors, of tumour biological features such as K-*ras* mutations and p53 overexpression, in assessing the prognosis of completely resected NSCLC patients. However, results of similar studies reported in the literature are conflicting and therefore the use of these additional biological parameters in clinical practice should still be considered investigational. The value of these molecular markers to guide novel adjuvant targeted therapies is still unknown and should be explored in future studies.

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