Predictive Survival Markers in Patients with Surgically Resected Non-Small Cell Lung Carcinoma[1]

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ABSTRACT

Among patients with resected non-small cell lung carcinoma, about 50% will present a tumor recurrence. Thus, it would be of major importance to be able to predict and try to prevent these relapses by an active chemotherapy and/or radiotherapy.

In an attempt to answer this question, the tumors of 227 patients with a surgically resected non-small cell lung carcinoma were evaluated as follows: tumors were classified as squamous cell carcinoma ($n = 132$) or adenocarcinoma ($n = 95$), and tumor differentiation was evaluated for each type. Then, all tumors were classified in respect to their pathological TNM staging (WHO) and screened by immunohistochemistry for the detection of the expression of the following antigens: Bcl-2, A+B+H blood group antigens, c-erb-b2, p53, and Pan-Ras antigens. Furthermore, adenocarcinomas were screened for the presence of point mutations in Ki-Ras codons 1–31. Finally, the patient blood group was defined, and patient survival was analyzed using nonparametric tests and proportional hazard Cox models.

Using Kaplan-Meier survival curves, disease pathological TNM staging was shown to be a strong predictive factor of survival for both squamous cell carcinoma and adenocarcinoma. Patients with squamous cell carcinoma experienced fewer relapses than those with adenocarcinoma (42% *versus* 63%; $P = 0.0002$) and had a significantly better survival.

All evaluated antigens were more often present in squamous cell carcinoma than in adenocarcinoma except for Pan-Ras (three times more frequent in adenocarcinoma).

In patients with squamous cell carcinoma, only tumor staging had a significant prognosis value ($P = 0.01$).

In patients with lung adenocarcinoma, a well-differentiated tumor ($P = 0.009$) as well as a positive Bcl-2 staining ($P = 0.009$) and an A+B+H antigen tumor staining ($P = 0.024$) were associated with a better survival. In contrast, patients with a stage I or II disease and a p53-positive tumor staining and patients with the O blood group ($P = 0.01$) had a shorter survival. Interestingly, no relation with patient survival was related to c-erb-b2 and Pan-Ras staining. Finally, 12 point mutations were found out of 81 tumors (15%) evaluated for Ki-Ras codons 1–31; they involved codon 12 but also 8, 14, and 15 without any relationship to survival.

In respect to lung adenocarcinoma, using Cox proportional hazard models stratified on tumor staging, the following markers were shown to be related to survival: (*a*) Independent markers of longer survival (*i.e.,* high histological degree of tumor differentiation and positive Bcl-2 and A+B+H blood group antigen expression by tumor cells); and (*b*) Independent markers of shorter survival (*i.e.,* O blood group for all patients and p53 tumor staining in patients with stage I and II diseases).

This study suggests that, in patients who undergo surgery for lung adenocarcinoma, the presence or absence of these criteria could be used to define a subset of patients who may benefit from a more specific follow-up.

INTRODUCTION

Lung cancer is the first cause of death by cancer in developed countries, and its incidence is steadily rising in the world. The WHO classification separates NSCLC[4] from SCLC. Among NSCLC, the incidence of adenocarcinoma is regularly increasing in comparison to squamous cell carcinoma. Although SCLC is usually treated by chemotherapy with or without radiotherapy, the best therapeutic opportunity for patients with NSCLC is surgery (1, 2). Indeed, a complete surgical resection remains the only real hope for cure. However, among patients who undergo surgery, about one of two will present a tumor recurrence. Thus, it would be of major importance to be able to identify these relapsing patients and to try to prevent a relapse

[4] The abbreviations used are: NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; pTNM, pathological TNM; RR, relative risk; CI, confidence interval.

by an active chemotherapy and/or radiotherapy. This concept explains the need to describe new prognostic factors for these patients. The pTNM staging is obviously one prognostic factor, but it is not sufficient. In view of the exploding knowledge about lung cancer biology, an active research is presently directed toward the evaluation of new cellular and/or molecular biological markers present in the tumor cells as prognostic factors and/or potential markers for early detection and/or possible targets for therapy. Several studies tend to integrate, in this respect, the present knowledge related to preneoplastic lesions (3, 4), but the results are presently conflicting (5). In this context, the study of a large number of patients with resected NSCLC was undertaken. All subjects underwent diagnosis, surgery, and follow-up in our institution. The classical clinicopathological features, pTNM, several biomarkers, and survival were recorded. The evaluated biomarkers were chosen according to our own experience, and they are among the most often reported ones in the literature as of prognostic importance: oncogenes (Bcl-2, c-erb-b2, Ki-Ras), the tumor suppressor gene p53, and also patient blood group type and blood group antigen expression on tumor tissue because these last two markers were also previously suggested to be related to lung cancer prognosis.

MATERIALS AND METHODS

Population Characteristics. All patients were consecutively referred to the Fédération Médico-Chirurgicale de Pneumologie (CHU de Nancy, Vandoeuvre-lès-Nancy, France) and were operated on by the same surgeon (J. B.) between November 1988 and May 1996. There were 105 primary adenocarcinoma and 141 squamous cell carcinoma patients. None of them had evidence of distant metastatic disease at diagnosis, except for four patients with solitary brain metastasis removed before lung surgery. Recorded patient clinical features included: age, sex, place of birth, occupation, blood type, smoking history, occurrence of previous cancers, presenting symptoms, type of surgery, and additional treatments. In this respect, after surgery, 3 patients were treated by chemotherapy, 105 by radiotherapy, and 7 by radio and chemotherapy. Patient survival was measured in months from the day of surgery until the date of death or the end of January 1998. pTNM grading and tumor typing were done according, respectively, to the recommendations of the American Joint Committee for Cancer Staging (6) and the WHO classification (7). Two pathologists (B. M. and J-M. V.) blindly reviewed each tumor in respect to tumor differentiation. A well-differentiated adenocarcinoma was characterized by a pattern with an acinar or papillary organization, or a bronchioloalveolar aspect. The remaining adenocarcinomas, such as those with a solid pattern, were classified as poorly differentiated. For squamous cell carcinoma, a well-differentiated tumor typically showed keratinization and/or intercellular bridges. Due to an invasion by cancer cells of the resection margin, 10 adenocarcinomas and 9 squamous cell carcinomas were removed from the study; thus, 227 patients were fully evaluated.

Immunohistochemistry Studies. All reagents were from Sigma (St. Quentin Les Yvelines, France) unless there were other specific indications. For each antibody, the specific working conditions were determined in preliminary experiments. Serial dilutions of antibodies and various experimental

procedures were tried, including each time negative control of the tumor. Then, 10 tumors were tested together in each of the following experiments, first for the detection of the ki-67 antigen (a biological marker related to cell proliferation used as a control) and next for the detection of the other antigens. In all experiments, one section of each of the 10 tested tumors was used as a negative control.

The procedure was as follows: consecutive representative sections of tumor samples embedded in paraffin were deparaffinized in toluene, rinsed in ethanol, and blocked for 30 min with 0.6% hydrogen peroxide in methanol. They were then rinsed again in ethanol and further washed twice in TNT [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% v/v Tween 20]. Antigen demasking was carried out in a pressure cooker in which the sections were immersed during 5 min in boiling 1.8 mM citric acid, 9 mM sodium citrate (pH 6.0) before cooling in running tap water and washing in TNT. All sections were then incubated overnight with the specific antibodies prepared in TNT. A (26A2), B (95.3), and H (AB5) antibodies (Pasteur diagnostic, Marne La Coquette, France) were respectively diluted at 1/50, 1/50, and 1/75; c-erb-b2 (CL-CB 11) at 1/100; p53 (DO 7) at 1/200 (both from Novacastra, Newcastle-upon-Tyne, England); Bcl-2 (124) at 1/40 (Dako, Les Ulis, France); Ki-67/HIB-1 at 1/200 (Immunotech, Lumigny, France); and Pan-Ras (Ab-2) at 1/200 (Oncogene Science, Manhasset, MA). All sections were then washed twice for 5 min in TNT and further incubated for 30 min in TNT containing 0.3% albumin w/v. Between each further incubation, two consecutive 5-min washes in TNT were performed. The second biotinylated goat anti-mouse antibody (dilution in TNT: 1/150, Dako) was applied for 30 min. A third 30-min incubation in a TNT solution of peroxidase-conjugated streptavidin (dilution:1/250, Dako) was followed by a fourth incubation with a tyramide-biotin conjugate as prepared by Plenat *et al.* [Ref. 8; dilution: 1/100 in 200 mM Tris-HCl (pH 8.8), 10 mM imidazole containing 1 μl/ml H_2O_2]. A fifth incubation repeated exactly the third incubation described above. The revelation was performed in 50 mM Tris-HCl (pH 7.6) containing diaminobenzidine tetrahydrochloride, 0.6 mg/ml, Brij 35, 0.29 ml, saponin, 10 ml, Tween 20, 0.05 ml, and 0.1% H_2O_2. The sections were finally counterstained in H&E and mounted for microscopic evaluation. Negative controls using isotype-specific polyclonal mouse immunoglobulins instead of primary antibodies showed no evidence of staining. The scoring and interpretation were simultaneously made by the two pathologists without knowledge of the clinical data. Bcl-2, Pan-Ras, c-erb-b2, and A+B+H blood group staining of tumor cells was defined as either present (with a granular staining in cytoplasm and/or cytoplasmic membrane) or absent. p53 staining was graded positive when seen in at least 30% of 1000 tumor cells counted with an optical grid.

Sequencing of Ki-Ras Codons 1–31. As soon as they were removed, the tumors were kept on ice until their arrival in the laboratory. At the same time, a paired piece of healthy parenchyma was also collected from a tissue as further away as possible from the tumor. The specimens were snap frozen in liquid nitrogen and stored at −80°C until processing. From each, DNA was prepared after tissue proteinase K digestion, phenol extraction, and ethanol precipitation as usual. The puri-

fied DNA was then aliquoted in sterile water at 1 mg/ml and kept at 4°C.

Single-stranded PCR products were generated only from adenocarcinoma DNA samples by asymmetric PCR using a pair of primers KA: GACTGAATATAAACTTGTGGTAGT and KB: CTATTGTTGGATCATATTCGTCC. All reactions were performed in 100 μl containing 10 μl of PCR mix, 200 mM of each deoxynucleotide triphosphate, 0.01 mM KB, 0.05 mM KA, 0.1 mg DNA, and 2 μl of Taq polymerase (Appligene, Strasbourg, France). The PCR consisted of 40 cycles of 45 s at 91°C, 1 min at 60°C, and 1 min at 70°C, including a hot start and a final 10-min extension step. The asymmetric PCR products were ethanol-precipitated at −20°C, rinsed with a 70% ethanol solution, and vacuum-dried for 1 h. Using KB for sequence priming, the single-stranded products were then sequenced by the dideoxy chain termination method with a Sequenase 2.0 kit and an (^{35}S)-labeled deoxyadenosine triphosphate (Amersham, Les Ulis, France) following the manufacturer's protocol. The sequence reactions were resolved by gel electrophoresis in 6% polyacrylamide denaturing gels. When a mutation was observed, the paired healthy parenchyma was analyzed in a similar fashion. The mutations were furthermore confirmed by sequencing following the Sequenase protocol of symmetric PCR products generated from the found mutated DNA samples as above but using 0.01 mM of each KA and KB, 1 ml of Taq polymerase, and only 28 cycles of PCR. The PCR products were first cloned in the pGEM-T Vector System (Promega, Charbonnières, France), and 30% of the recombinant colonies were then systematically sequenced using KB for sequence priming.

Statistical Analysis. The primary goal of this study was to determine separately for each cancer type the predictive significance of each parameter on the overall survival of the patients. All statistical analyses were done on the subset of 227 patients with complete tumor resection (resection margin free of tumor cells), stratified on tumor stage. Due to the very low number of patients with stage IV (two squamous cell carcinomas, four adenocarcinomas), they were pooled with stage IIIB patients. Patient survival was described by a standard Kaplan-Meier estimate stratified on disease staging. If a patient died during the follow-up, patient survival time was censored at the time of death. Medical history, clinical examination, and radiological evaluation were used to determine whether death resulted from recurrent cancer (relapsing patients) or from any other cause. Survivors were defined as free from clinical and radiological local or distant relapse at the end of the follow-up.

The predictive significance of each parameter was then tested univariately in a nonparametric fashion by a generalized Wilcoxon rank test conducted at the 5% level of significance ($P < 0.05$). These analyses were carried out using the Proc Lifetest from the SAS software (SAS Institute, Cary, NC). To quantify any relationship between evaluated parameters, patient survival, and tumor stage, and because no correlation was observed between evaluated parameters and survival in squamous cell carcinoma, a Cox proportional hazard model stratified on tumor stage was used to assess the simultaneous contribution of the different covariates only in adenocarcinoma. The following variables were considered for entering the model: age, sex, tumor staging, smoking history, extent of surgery, positive or negative detection of each tested antigen (except for ki-67),

blood type, and presence of Ki-Ras mutation. The Cox models were carried out using Proc Phreg from the SAS software (SAS Institute) and were developed in a forward fashion based on changes in log-likelihood. Interactions between tumor staging and the different parameters were considered. Possible confounding by the other factors was systematically assessed. The results of the Cox models are given as RR by which the hazard of relapse is multiplied when the factor is present *versus* when it is absent.

RESULTS

Population Description. No patient was lost to follow-up (18–109 months). The main patient characteristics are detailed in Table 1: the male:female ratio was 126:6 in squamous cell carcinoma and 83:12 in adenocarcinoma. Patients with squamous cell carcinoma were slightly older than those with adenocarcinoma (62 *versus* 60 years; $P = 0.21$). Smoking was significantly heavier in squamous cell carcinoma than in adenocarcinoma (43 ± 21 *versus* 37 ± 22 pack/year; $P = 0.04$). In the squamous cell carcinoma group, two males and one female had never smoked, and 58 had quit smoking about 7 ± 1 years before tumor resection. In the adenocarcinoma group, 10 patients had never smoked (4 males and 6 females), and 48 had quit smoking about 9 ± 8 years before surgery. A history of previous cancer was found in 19% of squamous cell carcinoma patients: 8 lung cancers, 10 orolaryngeal cancers, 3 squamous cell skin cancers, and 4 varied primaries. Among adenocarcinoma patients, 15% had had a previous cancer: two lung cancers, six orolaryngeal cancers, three squamous cell skin carcinoma, and three varied primaries resected >15 years before surgery for lung cancer. Preoperative diagnostic biopsies were obtained in 73% of squamous cell carcinomas and 52% of adenocarcinomas due to the fact that these last tumors are more frequently distal. Surgical resection was more extensive (pneumonectomy) for squamous cell carcinoma than for adenocarcinoma: 55% *versus* 32%. Postoperative deaths were more frequent in squamous cell carcinoma (11.6%) than in adenocarcinoma (6.3%) but were not related to the overall survival ($P = 0.1$). Patients with squamous cell carcinoma tended to die more frequently from other causes than lung carcinoma (22% *versus* 15%; $P = 0.28$). All patients with stage III and IV diseases had the same complementary radiotherapy if they survived for at least 60 days after surgery.

As a first step, patient survival was evaluated with respect to pTNM staging using Kaplan-Meier survival curves (Fig. 1) for both squamous cell carcinoma and adenocarcinoma. Low disease staging was found to be a strong predictive factor of a longer overall survival ($P = 0.01$) in both groups of patients. When each group was independently evaluated, a high pTNM staging was still a pejorative prognosis factor (stage I: $P = 0.07$; stage II: $P = 0.15$; stage III: $P = 0.006$; stage IV: $P \leq 0.05$). In squamous cell carcinoma, about two of three patients with stage I and II were alive at, respectively, 60 months and 45 months after surgery, whereas about only one-third of stage III and IV patients were still alive at about, respectively, 42 months and 22 months after surgery. In adenocarcinoma, 40% patients with stage I survived for 60 months, 20% with stage II for 65 months,

Table 1 Population characteristics

| | Tumor staging: pTNM | | | | |
	I	II	IIIA	IIIB + IV	All stages
Squamous cell carcinoma					
Male[a]	42	24	43	17	126
Female[a]	3	1	0	2	6
Age (yr)	62.96 ± 9.30	60.16 ± 9.50	61.98 ± 9.65	60.74 ± 9.31	61.79 ± 9.41
Tobacco (pack-yr)	45.18 ± 22.35	40.32 ± 20.83	43.12 ± 20.83	37.47 ± 21.58	42.55 ± 20.49
Nonrelated deaths[a]	11	5	11	2	29 (21.97%)
Relapses[a]	10	8	26	11	55 (41.67%)
Survivors[a]	24	12	6	6	48 (36.36%)
Length of survival (mo)	41.55 ± 27.62	38.68 ± 29.52	27.71 ± 27.95	21.95 ± 23	33.68 ± 28.23
Adenocarcinoma					
Male[a]	39	9	22	13	83
Female[a]	7	2	2	1	12
Age (yr)	63.57 ± 9.26	58.45 ± 8.13	58.50 ± 9.45	54.50 ± 9.03	60.36 ± 9.00
Tobacco (pack-yr)	40.07 ± 25.69	30.64 ± 21.91	37.35 ± 13.03	34.29 ± 19.92	37.44 ± 21.84
Nonrelated deaths[a]	6	2	5	1	14 (14.73%)
Relapses[a]	24	6	18	12	60 (63.16%)
Survivors[a]	16	3	1	1	21 (22.11%)
Length of survival (mo)	35.5 ± 24.90	33.55 ± 26.32	19.64 ± 18.95	28.64 ± 29.48	30.26 ± 24.95

[a] In number of patients.

10% with stage IIIA for 50 months, and 10% with stage IIIB for 65 months.

Relapses were significantly less frequent in squamous cell carcinoma than in adenocarcinoma: 42% *versus* 63% ($P = 0.0002$). And this explains, at least in part, the best overall survival of these patients (36% *versus* 22%; $P \leq 0.05$). This observation led us to separately analyze patients with squamous cell carcinoma *versus* patients with adenocarcinoma.

ABO blood typing was similar for squamous cell carcinoma and adenocarcinoma: O group: 41.66% *versus* 39.36% ($P > 0.05$); A group: 40.90% *versus* 40.42% ($P > 0.05$); B group: 13.63% *versus* 13.8% ($P > 0.05$); and AB group: 3.78% *versus* 6.38% ($P > 0.05$).

Histology and Immunohistochemistry. Squamous cell carcinomas were more often positively stained than adenocarcinomas for the following antigens: Bcl-2 (30% *versus* 19%), c-erb-b2 (26% *versus* 13%), p53 (69% *versus* 47%), and A+B+H (51% *versus* 31%). Only for Pan-Ras antibodies were adenocarcinomas more frequently stained than squamous cell carcinomas: 35% *versus* 12%.

In squamous cell carcinoma patients, besides pTNM, no other evaluated parameter had a significant predictive value in respect to survival in the univariate analysis, and no parameter was related to tumor staging or to any other parameter (Table 2). However, it is worth noting that tumor cell staining with A+B+H antibodies (42% tumors) was related to a tendency to have a better prognosis ($P = 0.07$).

In marked contrast, in adenocarcinoma patients, a histologically differentiated tumor was associated with a longer survival ($P = 0.009$; Table 3). Interestingly, while in all adenocarcinoma sections, Bcl-2 was always detected in relation to stromal lymphocytes, and 19% of tumors also showed an associated cytoplasmic membrane staining of the tumor cells; such a positive staining was associated with a longer survival ($P = 0.009$). No other correlation between Bcl-2-tumor-cell-positive staining and any other parameter was observed.

In all adenocarcinoma tumor sections, endothelial cells were positively stained by the mixture of A+B+H antibodies, but a positive tumor cell staining was observed in only 30% of cases, and this staining was associated with a better prognosis ($P = 0.024$). When looking at blood groups, patients with the O blood group had a shorter survival ($P = 0.01$). Interestingly, among 36 patients with the O blood group, 33 had an A+B+H-unstained tumor (in comparison, 23 of 55 squamous cell carcinoma tumors of patients with the O blood group were A+B+H-unstained).

In adenocarcinoma patients, p53 was detected in tumor cells in 47% of the cases and was associated with a tendency to have a poorer survival ($P = 0.07$). When early stages (stage I + stage II) were isolated, p53 tumor staining was associated with a shortened survival (RR, 4.56; 95% CI, 1.89–11). In contrast, positive p53 staining in the late stages of adenocarcinoma (stages IIIA + IIIB and IV) was not related to survival (RR, 0.50; 95% CI, 0.20–1.2).

A positive tumor cell staining with the Pan-Ras antibody was observed in 33% of the adenocarcinomas, and no significant survival pattern was associated with this staining ($P = 0.4$).

Finally, 13% of the adenocarcinomas were positively stained (overexpression) with the c-erb-b2 antibody, but this staining did not affect patient survival ($P = 0.34$).

Mutations in Ki-Ras Codons 1–31 in Lung Primary Adenocarcinoma. Among the 81 tumors evaluated for point mutations in Ki-Ras codons 1–31, 12 gene mutations were found. In respect to codon 12, six point mutations of the normal GTT (glycine) sequence to GTG (cysteine) were observed, a mutation to GTC (valine) was observed twice (Fig. 2, *panels 1, 2,* and *3*), and one glycine-to-cysteine mutation was associated with a codon 8 TCC-to-TAC mutation (Fig. 2, *panel 4*). Three other point mutations were observed: twice at codon 14 (GCT to GAT) and once at codon 15 (TAC to TAT; Fig. 2, *panels 5* and *6*). In all cases, no mutation was observed in the paired normal parenchyma.
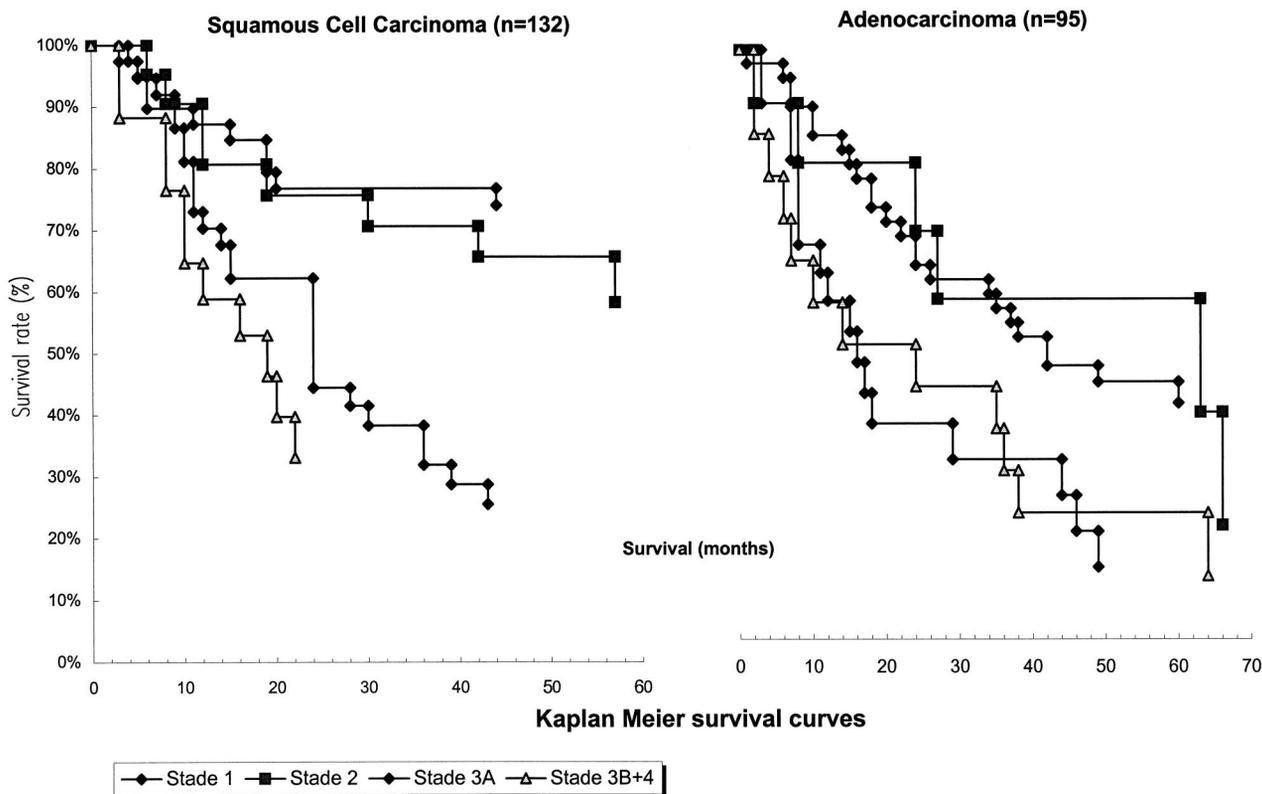
Fig. 1 Kaplan-Meier survival curves. Survival curves were constructed using the Kaplan-Meier classical method, and they included the 227 patients. Cumulative survival is expressed as a percentage, and survival is expressed in months as described in "Materials and Methods."

The sequencing of the cloned symmetric PCR products confirmed in each case the mutations found within the asymmetric PCR products (Fig. 2, *panels 1, 2,* and *3*). Only three patients had both a positive Pan-Ras tumor staining and a Ki-Ras point mutation: once a glycine-to-cysteine mutation, once a codon 14 mutation, and once a codon 15 mutation. Ki-Ras mutations were found in early or late stage diseases (as well as in relapsing patients and in one long survivor) but only in smokers. The Ki-Ras mutations were not predictive markers of a shorter survival ($P = 0.26$).

Cox Models. In the Cox proportional hazard models stratified on tumor staging (Table 4), the stepwise selection procedure resulted in the inclusion of different markers, demonstrating that a high histological degree of tumor differentiation, a positive Bcl-2 tumor cell staining, and an A+B+H-positive tumor cell staining were three independent indicators of longer survival, whereas an O blood group was a pejorative marker in model A. Two alternate models (B and C) with interaction are also shown in Table 4. They demonstrate a shorter survival related to a p53 tumor positive staining in patients with limited (stages I and II) diseases.

DISCUSSION

An exhaustive review of the literature on lung cancer predictive markers is beyond the scope of this report. Only studies directly relevant to this work will be discussed. The 5-year survival rate for all NSCLC combined stages is only 13%, but it reaches 47% for surgically treated early stages. Thus, the presence of a resectable disease is the most commonly used prognostic marker in NSCLC (9). However, one-half of the patients with resected stage I and II tumors will relapse, suggesting different levels of tumor virulence among the same pTNM cases. A large variety of factors has been evaluated to define, among these patients, those with a bad prognosis. Strauss (3) recently reviewed them, underlying the numerous but often conflicting results. The importance of the tumor size and of a careful tumor staging has been constantly recognized as an important prognosis marker, as well as histological typing: patients with a squamous cell carcinoma have a significant lower relapse rate than those with adenocarcinoma (3). A longer survival is also associated with a differentiated adenocarcinoma (3); no clear result for squamous cell carcinoma has been shown in this respect.

In a study of 271 consecutive patients with stage I NSCLC, Harpole *et al.* (10) reported that the male sex was a significant univariate predictor of early recurrence. We also found, in the univariate analysis, that females with resected adenocarcinoma were more likely to survive longer than males, but this was not confirmed after the Cox model building due to the small number of women in this study.

The antigens evaluated in this study are the most often reported in the literature as of prognostic importance, mainly

Table 2 Squamous cell lung carcinoma: predicative significance of each parameter

Tumor cell staining	Covariates					
	Death from other causes	Survivors	Relapses	Survival (mo)	No. of patients	P
Differentiation[a]						
+	16	31	30	39.90 ± 30.50	77	0.30
−	13	17	25	24.97 ± 22.16	55	
Bcl-2[b]						
+	13	12	14	30.02 ± 28.04	39	0.99
−	16	35	41	34.61 ± 27.86	92	
ABH antigens[b]						
+	16	27	25	37.41 ± 29.55	68	0.074
−	13	21	30	29.71 ± 26.40	64	
p53[b]						
+	19	34	38	34.73 ± 28.45	91	0.66
−	10	14	17	31.34 ± 27.93	41	
Pan-Ras[b]						
+	2	8	6	39.28 ± 26.78	16	0.38
−	27	40	49	32.91 ± 28.45	116	
C-erbB2[b]						
+	7	12	16	32.45 ± 27.32	35	0.83
−	22	36	39	34.12 ± 28.67	97	
Blood types						
O	9	24	22	38.71 ± 26.89	55	0.20
B	4	6	8	37.22 ± 29.97	18	0.74
A	15	14	25	25.23 ± 26.46	54	0.08
AB	1	4	0	56.80 ± 34.79	5	0.056
Sex						
Male	28	53	45	33.70 ± 28.4	126	0.66
Female	1	3	2	34.00 ± 27.7	6	

[a] Tumor histological subtyping: +, well-differentiated; −, moderately or poorly differentiated.
[b] Immunohistochemistry: +, positive tumor staining; −, negative tumor staining.
[c] Univariate Wilcoxon rank test pooled over tumor stage.

due to their overexpression. To avoid patient recruitment bias, a large patient population of consecutive and carefully staged patients with complete tumor resection was included. All tumors were diagnosed, treated, and followed up in the same institution by the same medical team with the same surgeon, and the biological studies on tumors were done by the same research team; furthermore, to increase the performance of immunohistochemistry, a tyramide-biotin amplification step was carried out, whereas ki-67 antigen detection was used as an internal control of an efficient antigen retrieval for each tumor. This was important to demask the antigens in tissues fixed in formaldehyde for the oldest archived biopsies (from 1988 to 1990).

Bcl-2 is the acronym of the B-Cell Lymphoma/Leukemia-2 gene mapping on chromosome 18q21. The involvement of Bcl-2 through apoptosis dysregulation in cancer development is based on the observation that genetic translocation or transgenic manipulation causing overexpression of Bcl-2 may induce the development of lymphomas. Bcl-2 is abnormally expressed in >50% of SCLCs with no prognosis value (11), but Pezzella *et al.* (12) showed that a lower malignancy was associated with a Bcl-2-positive staining in NSCLC in patients with stage I and II tumors: 20 of 80 squamous cell carcinomas and 5 of 42 adenocarcinomas. In our study, 39 of 92 squamous cell carcinomas were positively stained as well as 18 of 77 adenocarcinomas. Ohsaki *et al.* (13) and Fontanini *et al.* (14) confirmed Pezzella's findings in NSCLC. The association of Bcl-2 overexpression in adenocarcinoma with a better survival is surprising because Bcl-2 is thought to be an oncogene with antiapoptotic activity.

However, Bcl-2 phosphorylation results in the loss of its antiapoptotic function; therefore, high phosphorylated Bcl-2 levels may not prevent apoptosis. Unfortunately, phosphorylation is not detectable by immunohistochemistry. Another possible explanation is related to the involvement of other members of the ever-growing Bcl-2 family, such as Bax (a gene that encodes a dominant inhibitor of Bcl-2) and Bcl-x, because Bcl-2 is not *per se* the critical factor of susceptibility to an apoptotic stimulus. Furthermore, the Bcl-2:Bax ratio may be important (15). Finally, a potential role of Bcl-2 as an inhibitor of tumor angiogenesis has been recently described in NSCLC (16). Taken together, these studies and our work indicate that Bcl-2-positive immunostaining can be used for the everyday assessment of adenocarcinoma patients. This proposal is strengthened by the fact that the test is inexpensive and easy to perform, and the constant staining of stromal lymphocytes is an internal quality control.

A, B, and H blood group antigens are expressed, not only by erythrocytes, but also by several other tissues, including some epithelial and endothelial cells. These antigens also exist as water soluble molecules whose expression is determined by the secretor locus. A precursor oligosaccharide is first fucosylated by a (1, 2) fucosyl transferase encoded by the H blood group locus (or the secretor locus). The resulting H molecule is then the substrate of the glycosyltransferases encoded by the A or B blood group loci, leading to, respectively, the A and B blood group antigenic determinants (17). These antigens and other blood group carbohydrates have a biological relevance

Table 3 Lung adenocarcinoma: predictive significance of each parameter

Tumor cell staining	Death from other causes	Survivors	Relapses	Survival (mo)	No. of patients	*P*
			Covariates			
Differentiation[a]						
+	4	25	31	36 ± 25	60	0.009
−	7	6	22	20.56 ± 22.69	35	
Bcl-2[b]						
+	4	7	7	41.60 ± 28.91	18	0.009
−	10	14	53	27.60 ± 23.35	77	
ABH antigens[b]						
+	6	6	17	33.19 ± 22.44	29	0.024
−	8	15	42	29.30 ± 26.09	65	
p53[b]						
+	5	5	34	27.53 ± 24.30	44	0.076
−	9	16	25	32.50 ± 25.75	50	
Pan-Ras[b]						
+	5	7	20	22.81 ± 18.11	32	0.43
−	9	14	39	23.85 ± 19.27	62	
Ki-RAS mutations						
+	2	1	4	24.13 ± 18.85	12	0.34
−	12	20	56	18.56 ± 18.19	83	
C-erbB2[b]						
+	2	4	6	35.05 ± 32.67	12	0.34
−	12	17	54	29.56 ± 23.80	83	
Blood types						
O	6	7	24	23.09 ± 25.52	37	0.018
B	1	4	8	42.38 ± 28.03	13	0.32
A	5	6	27	31.58 ± 20.02	38	0.39
AB	2	3	1	33.17 ± 32.52	6	0.26
Sex						
Male	12	17	54	28.17 ± 24.36	83	0.0014
Female	2	4	6	44.69 ± 25.21	12	

[a] Tumor histological subtyping: +, well-differentiated; −, moderately or poorly differentiated.
[b] Immunohistochemistry: +, positive tumor staining; −, negative tumor staining.
[c] Univariate Wilcoxon rank test pooled over tumor stage.

that exceeds their roles in blood transfusion. Lee *et al.* (18) reported that blood A group antigen expression by tumor cells in NSCLC was strongly correlated with a longer survival when tumor samples were examined at the earliest stage of development (61% of positively stained tumors). Matsumoto *et al.* (19) found a correlation between the metastatic potential of NSCLC and the loss of blood group antigen expression (46% of positively stained tumors). In our study, a positively stained tumor was associated with a tendency to a better prognosis in squamous cell carcinoma and with a clear-cut better prognosis in adenocarcinoma. The A and B antigen deletions observed in most adenocarcinoma could be related to the loss of the short arm of chromosome 9 frequently observed in NSCLC (20) because the transferase genes are located on 9p34. Furthermore, the fucosyl transferase III, encoded by the Lewis blood group located on 19p13.3, may use the secretor H determinant to synthesize the Lewis blood group antigens. The Sialyl Lewis antigen is a ligand for the E-selectin receptor, and its expression has been related to the metastatic potential of cancer cells in some studies but not in others (for review, see Ref. 21). In adenocarcinoma patients with the O blood group, the accumulation of transformed H determinants or Sialyl Lewis antigens may explain our findings of a shortened survival in these patients. However, H determinant transformation is difficult to prove because we used an anti-H monoclonal antibody without a defined antigen specificity. Moreover, a comparison with the

previous findings of Lee *et al.* (18) are difficult because Ulex Europaeus agglutinin I was used in their study for H antigen detection. Other fucosyl transferases may participate in the biosynthesis of Sialyl Lewis antigen and thus play a role in the E-selectin receptor-mediated modulation of the metastatic potential. Interestingly, the survival of patients whose adenocarcinoma strongly expressed fucosyl IV and VI transferases was shown to be significantly shortened (22). Our findings confirm that, in lung adenocarcinoma, an O blood type is a pejorative marker, whereas a positive A+B+H antigen staining by tumor cells is associated with a better survival. In a similar fashion as for Bcl-2, this test is cheap and easy to perform, and the always positive staining of blood vessel cells can be used as an internal quality control.

Mutations of the P53 gene (mapping at 17p13) often result in the production of a p53 protein with an increased stability that explains a positive immunohistochemical staining in mutant cells, in contrast to cells containing the P53 wild type which, generally are not stained due to the shorter life of the wild p53 protein. Although the mutations resulting in p53 immunostaining are frequent enough to allow this technique to be of some practical value, a negative immunostaining does not rule out P53 mutations. Indeed, Casey *et al.* (23) demonstrated that DNA sequence analysis of all P53 exons with p53 immunostaining are both required to detect all known P53 alterations with a high concordance between the presence of P53 missense
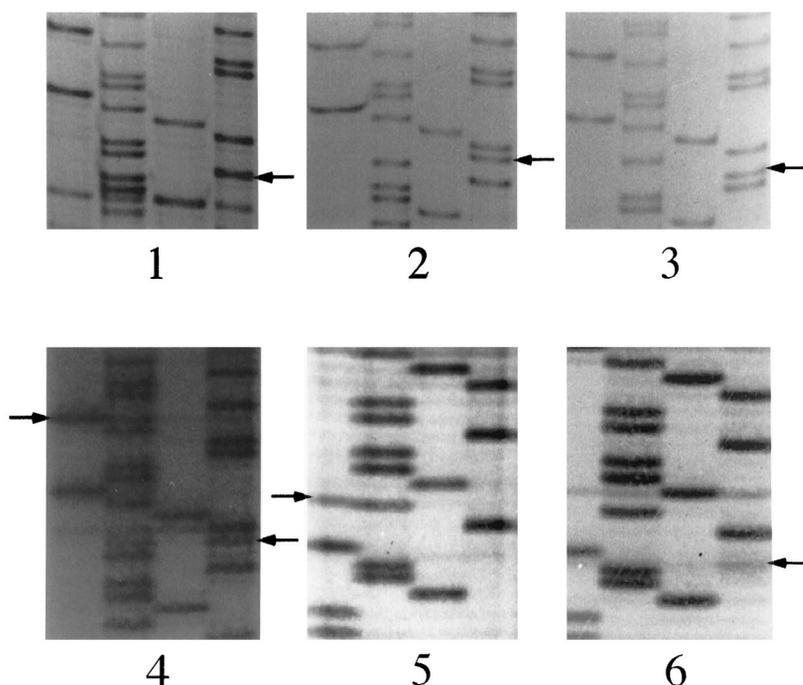
Fig. 2 Sequencing of *Ki-RAS* in adenocarcinoma. Sequencing of *Ki-RAS* cloned symmetric PCR products [*panel 1,* normal codon 12, GTT (glycine); *panel 2,* mutated codon 12, GTG (cysteine); *panel 3,* mutated codon 12, GTC (valine)] and sequencing of *Ki-RAS* asymmetric PCR products [*panel 4,* codon 8 mutation (TCC to TAC) + codon 12 cysteine mutation; *panel 5,* codon 14 mutation from GCT to GAT; and *panel 6,* codon 15 mutation from TAC to TAT].

Table 4 Lung adenocarcinoma: Cox proportional hazard models stratified on tumor staging

| | Covariates | | |
| | Univariate Cox model RR and 95% CI | Selected multiple Cox models with interaction: RR and 95% CI | |
	A	B	C
Differentiation[a]	0.41 (0.23–0.72)	0.33 (0.17–0.63)	0.41 (0.22–0.78)
Bcl-2[b]	0.41 (0.18–0.90)	0.32 (0.14–0.74)	0.33 (1.13–0.79)
A + B + H Antigens[b]	0.66 (0.37–1.19)		0.40 (0.21–0.77)
p53[b]	1.75 (0.99–3.08)	4.56 (1.89–11)[c]	4.50 (1.89–10.7)[c]
		0.50 (0.20–1.2)[d]	0.45 (0.18–1.13)[d]
Blood group	1.54 (0.90–2.63)	3.01 (1.61–5.64)	

[a] Well-differentiated tumors.
[b] Positively stained tumor cells.
[c] Stages I and II.
[d] Stages III and IV.

mutations and positive immunostaining. P53 mutations are known to occur in about one-half of all cancers, and likewise, in one-half of NSCLCs (24). The predictive survival value of these mutations is not formally due to conflicting published data (3). The tumors of about 46% of our patients with adenocarcinoma were positively stained for p53, and a pejorative prognostic value was associated with this staining in early stage diseases (stages I and II). These results are very close to those reported by Nishio *et al.* (25), but other results have been reported (21).

The c-erb-b2 proto-oncogene, located on chromosome 17 q21, encodes for a 185-kDa transmembrane protein having an intrinsic tyrosine kinase activity and sharing an extensive sequence homology with the epidermal growth factor receptor. In the normal lung, c-erb-b2 is expressed, at low levels, by ciliated cells, type II pneumocytes, and bronchial submucosal glands.

Furthermore, c-erb-b2 gene amplification and protein overexpression have been observed in a variety of epithelial tumor locations, including breast, bladder, and NSCLC. In NSCLC, c-erb-b2 protein overexpression has been linked to a poor prognosis, especially when associated with Ki-Ras mutations (26). However, other studies found opposite results (27). The overall interpretation of all published results is difficult because some authors distinguish a cytoplasmic staining from a membranous one, the cutoff values for positive results can vary, and finally, different antibodies have been used. We used the CL-CB11 antibody that recognizes the internal domain of the c-erb-b2 protein and mainly stains the cytoplasm on paraffin-embedded tissue sections. As recently shown with the same antibody (28), a positive tumor staining is not related to a shortened survival. In an extensive study performed by Pfeiffer *et al.* (29) on

cryostat lung cancer sections with an another antibody, the same lack of prognostic significance was observed.

The transforming potential of *RAS* oncogenes can be due to a single amino acid substitution of the Ras proteins related to cell growth regulation. Point mutations are found in ~30% human tumors. For Slebos *et al.* (30), Ki-Ras mutation is the single most important pejorative prognostic marker in lung adenocarcinoma after adjustment for disease staging. Several mutations have been reported, including codons 13, 18, and 61 but also codon 61 of *N-Ras*, and such mutations have been shown in 21% of squamous cell carcinomas and 14% of large cell carcinomas (31). Rosell *et al.* (32) found Ki-Ras mutations of codons 12, 13, and 61 in about 26% of adenocarcinomas. However, Reynolds *et al.* (33) reported that 86% of lung cancer DNA from smokers contained activated proto-oncogenes related to the *RAS* family; and 30–40% with a Ki-Ras point mutation. Furthermore, Clements *et al.* (34) found Ki-Ras mutations in the healthy parenchyma of patients with lung tumors, but also in the normal lung of smokers without malignancy. Differences in patient sampling and the techniques used to detect the mutations most likely account for some reported discrepancies among these studies; however, direct asymmetric PCR product sequencing and confirmation by classical PCR, as carried out in this study, although not an easy procedure for screening, remain the gold standard techniques for such studies. In respect to our work, codon 61 was not specifically analyzed for activating mutations, and this may contribute to the absence of correlation between Ras mutations and survival. Generally speaking, European groups tend to associate a significant prognostic value to Ki-Ras mutations, whereas United States studies suggest the type of mutation to be a better prognostic indicator than the simple presence of mutations (35). Obviously, studies with larger patient populations are needed to help answer this question.

The previously published Pan-Ras antigen expression analysis by immunohistochemistry yielded somewhat different results from ours: >70% of adenocarcinomas were found to be positive, and a strong staining was associated with more advanced diseases (36). In our study, a positive staining was less frequent and was not associated with a shortened survival either in squamous cell carcinoma or in adenocarcinoma. Furthermore, a positive staining was not related to a specific Ki-Ras-specific genotype, but it was mostly associated with *Ha-Ras* amplification previously detected (and published) by *in situ* hybridization in some tumors (37).

In conclusion, most previous reports on predictive markers of lung cancer usually emphasize only positive results and forget negative ones. The importance of this fact is well illustrated in this study in the case of squamous cell carcinoma. We suggest that, in lung adenocarcinoma, some molecular markers can be evaluated for the follow-up of the patient including: (*a*) histological tumor differentiation, (*b*) Bcl-2, (*c*) A+B+H blood group antigen, and (*d*) p53 tumor cell staining (but p53 immunostaining does not necessarily correlate with mutations in this tumor suppressor gene), as well as (*e*) O blood group type. This observation is confirmed by the report by Kwiatkowski *et al.* (38) of a prospective study of 244 stage I and II NSCLCs using some similar markers. Although their study, in comparison to ours, did not involve other disease stages, included large cell

carcinomas, did not involve other Ki-Ras mutations than those involving codon 12, and detected only A blood group antigen presence in carcinoma cells (with no details about patient blood types), their findings, in a Cox model, are closer to ours in respect to the good predictive value of Bcl-2 expression, tumor differentiation, with the presence of mucin, and early tumor staging. Furthermore, a shortened survival was associated with p53 expression by tumor cells.

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